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A phage cocktail approach for the control of iron bacteria from biofouled water supply wells

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A phage cocktail approach for the control of iron bacteria from biofouled water supply wells

by

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Thesis

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Dedication

This thesis is dedicated to my wife Ana and my son Tomás,

who understandingly put aside their needs so I could finish this work.

Acknowledgements

ABSTRACT

A phage cocktail approach for the control of iron bacteria from biofouled water supply wells

by

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The University of Texas at Austin, 2015

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Biofouling refers to the accumulation of biofilm and biogenic precipitates on wetted surfaces and is one of the major problems associated with groundwater production. Groundwaters rich in ferrous iron (Fe(II)) are particularly susceptible to biofouling due to the low energetic yield of Fe(II) oxidation, which forces iron-oxidizing bacteria to convert large quantities of soluble Fe(II) to insoluble ferric iron (Fe(III)) to sustain growth. Bacteria-specific viruses, known as bacteriophages or phages, are particularly promising for biofilm control given their specificity and ability to spread from one host cell to another. Phage therapy for biofilm control has been most successful with pure cultures and low-diversity bacterial communities. However, the applicability of phages to control mixed bacterial communities, in particular those of groundwater supply wells, remains largely unexplored. To assess whether phages constitute an effective alternative to treat well biofouling, the microbial communities of two biofouled wells were interrogated by 16S rRNA gene sequencing. Potential key species of iron bacteria (FeB) were identified and enriched by repeated subculturing in

Modified Wolfe and Winogradsky media. Mixed phage suspensions were extracted by filtration from activated sludge, and this suspension was enriched against bacterial enrichment cultures developed from well water inocula. Batch tests showed a decrease in cell density and an impairment in iron redox cycling on well enrichment cultures when exposed to phage suspensions. Future work includes testing the phage suspensions against mixed biofilms of FeB, characterizing the specificity of these phage suspensions, and evaluating the impact of the phage on iron redox cycling under other culture conditions. Overall, this work is a first step toward a sustainable rehabilitation approach for biofouled wells.

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LIST OF FREQUENTLY USED ACRONYMS

FAS   Filtered Activated Sludge - a mixed phage suspension

FeB   Iron-bacteria

FeOB   Iron-oxidizing bacteria

FeRB   Iron-reducing bacteria

IC   Iron Cycling

MW   Modified Wolfe

PMW   A phage suspension enriched in Modified Wolfe medium

PPa   A phage suspension enriched against *Pseudomonas aeruginosa*, a non-iron-bacterium

PSn   A phage suspension enriched against *Sphaerotilus natans*, an iron-oxidizing bacterium

PW7   A phage suspension enriched against well 7bacteria

PW9   A phage suspension enriched against well 9 bacteria

PWI   A phage suspension enriched in Winogradsky medium

W7   Well # 7

W9   Well # 9

WI   Winogradsky

1 INTRODUCTION

1.1 Water supply well biofouling

Groundwater comprises about 25% of the global fresh water (Alley, Reilly, and Franke 1999), making it vital to meeting humankind's water demands. Groundwater is used extensively in the United States (US), accounting for as much as 30% of public supply, 40% of irrigation, and 60% of livestock and mining activities (Kenny et al. 2009). In Texas alone, groundwater accounts for approximately 60% of the total water used by the state (Cruz and Alexander 2014).

A typical groundwater well consists of a stainless steel casing installed through the underlying stratigraphy to a water-bearing aquifer. One or more screening intervals installed within these water-bearing zones allow groundwater to be pumped into the well while filtering out solids. While larger suspended solids are prevented from entering the well column, bacteria can still pass freely through the screens. Surface-attached bacterial communities known as biofilms can develop, with concomitant accumulation of biomass and microbial products around and within the screening interval (Taylor, Lange, and Lesold 1997). This process is known as biofouling and it can reduce the yield of a well up to 90% (Borch, Smith, and Noble 1993); a biofouled well often requires several treatment techniques to restore its productivity.

Conventional well rehabilitation involves physical/chemical processes including application of acids and disinfectants, heating, shockwaves, and scouring techniques, but these methods have varying levels of effectiveness, are often temporary fixes, and can be costly for water suppliers (Smith and Comeskey 2009). Some water suppliers spend several million dollars annually redeveloping, reconditioning, and replacing wells compromised due to biofouling issues (Smith and Comeskey 2009).

1.2   Iron bacteria and well biofouling

One particularly troublesome biofouling situation occurs in groundwater supplies with high levels of ferrous iron (Fe(II)) that is metabolized by iron-oxidizing bacteria (FeOB) (Bird, Bonnefoy, and Newman 2011). FeOB can be autotrophic or heterotrophic, depending on their carbon source, but in both cases, FeOB couple Fe(II) oxidation with oxygen reduction to water for energy conservation (Emerson and Weiss 2004). However, with an E_0' of approximately +0.4 V at pH 7 (Madigan et al. 2012), the energy yield of aerobic iron oxidation is low. Therefore, FeOB must convert large quantities of Fe(II) to ferric iron (Fe(III)) to sustain growth.

Fe(II) is stable and soluble below pH 5 but is rapidly oxidized by oxygen at circumneutral pH (Stumm and Morgan 1970). However, if the dissolved oxygen concentration is low, FeOB might effectively compete with, and even outcompete, this abiotic Fe(II) oxidation (Emerson and Weiss 2004); due to groundwater pumping activity, these favorable conditions with low dissolved oxygen are more likely to occur at the screen intervals of operating groundwater supply wells (Taylor, Lange, and Lesold 1997), and that is where FeOB tend to accumulate. Both the direct metabolic oxidation of Fe(II) to insoluble Fe(III) hydroxides and the inadvertent precipitation of Fe(III) oxides on microbial surfaces (Konhauser 1998) contribute to the accumulation of biogenic iron oxides (BIOs); these BIOs, along with biomass, eventually clog the wells. It has been shown that these BIOs are quite homogeneous in their mineralogical composition, even though they might come from very diverse microbial communities (Vollrath et al. 2013).

BIO-forming communities normally contain FeOB, with the autotrophic *Gallionella* and the heterotrophic *Crenothrix* often being the most abundant genera; additionally, iron-reducing bacteria (FeRB) and various aerobic heterotrophs are frequently present (Kato et al. 2013; Quaiser et al. 2014). It has been proposed that the FeRB and heterotrophic bacteria establish a collaboration that expands the FeOB niche (Elliott et al. 2014), and, as a consequence, FeOB might be found in fully oxygenated,

low Fe(II) environments that they would be otherwise unable to colonize (James and Ferris 2004). Therefore, a more holistic approach that considers FeOB, FeRB, and aerobic heterotrophic bacteria must be adopted in studies of iron-related well biofouling.

1.3 Bacteriophages as a means to control well biofouling

A possible strategy, and one that remains largely unexplored, is the use of bacteriophages, or simply phages, to kill the bacteria responsible for biofouling. Phages are viruses that specifically infect bacteria (Adams 1959); according to their reproductive strategy, they can be either lytic, where they readily multiply and lyse their host after infection, or lysogenic, where they integrate into the host genome and remain latent until the appropriate conditions for reproduction arise (Adams 1959).

Phages offer particular promise for the control of unwanted bacteria in a variety of environments: agriculture (Fujiwara et al. 2011), biomedicine (Morello et al. 2011), the food industry (García et al. 2008), and wastewater treatment (Vanysacker et al. 2014). However, attempts to use phages to control biofouling in drinking water wells have been very limited. While most well-reconditioning research has focused on improving conventional physical/chemical treatment methods, only a handful of studies have investigated the use of bacteriophages for well-reconditioning (e.g. Gino et al. 2010). Overall, the use of phage therapy for bacterial control generally has been assessed with single-species or very low diversity communities.

1.4 Objectives

This study aimed to identify the microbial communities responsible for biofouling of water supply wells and to evaluate the applicability of a mixed phage therapy to control the bacterial consortia. To this end, iron bacteria (FeB), including both FeOB and FeRB, were enriched from biofouled well water and solids samples, and

these bacterial enrichments were used to enrich phage suspensions against them. The phage suspensions were then tested for their ability to reduce bacterial growth and impair bacterial iron redox cycling.

2 MATERIALS AND METHODS

2.1 Bacteria culturing and maintenance

2.1.1 Sources and description of bacteria

2.1.1.1 Biofouled well samples

Samples were provided by GSI Water Solutions, Inc. from wells with reported iron biofouling problems in two cities in Oregon. Samples were collected in sterile plastic vials and shipped overnight on ice to the University of Texas at Austin. Samples were kept at 4 °C, and aliquots were taken from the samples for microbial culturing and community analysis within one week. Details of sample collection are given below (sections 2.1.1.1.1 and 2.1.1.1.2), and sample descriptions are summarized in Table 1.

2.1.1.1.1 City of Fairview, Oregon

As part of well rehabilitation activities, several samples were collected. In June 2014, the City of Fairview took well #9 off line, the pump was connected to a column that resides within the well's casing. A GSI employee collected a groundwater sample in a sterile 1-L polyphenylene ether (PPE) bottle from a portion of the pump column below the well head. Solids that had accumulated in the same section of the pump column were scraped into the PPE bottle using a sterile nitrile glove.

2.1.1.1.2 City of Troutdale, Oregon

Samples of biomass and solids that had accumulated on pressure transducers within wells #6, 7, and 8 were collected in April 2014. Pressure transducers are used to monitor fluctuations in water levels and reside at depths above the well screening interval and above, but near, the pump intake depth. The transducers were removed from each well, and water from the well's sampling port was used to flush accumulated

solids/biomass from the transducer into a sterile PPE bottle. Nitrile gloves were worn during sampling activities.

Table 1. Description of water samples. W= Well; FH = Fire Hydrant. The numbers after W9 indicate sampling event.

Sample	Location	Date	pH	Fe(II) [mg/L]	Description
W9	City of Fairview	Jun-14	7.0	0.06	Water + BIOs
W6	City of Troutdale	Apr-14	7.2	0.2	Water + BIOs
W7		Apr-14	7.1		Water + BIOs
W8		Apr-14	7.3		Water + BIOs

2.1.1.2   Control strains

The autotrophic iron-oxidizer *Sideroxydans lithotrophicus* (ATCC® 700298) and the heterotrophic iron-oxidizer *Sphaerotilus natans* (ATCC® 13338) were used as FeOB positive controls. *Pseudomonas aeruginosa* was used as the negative control (non-FeB) for some experiments. The heterotrophic *S. natans* was selected because of its ability to grow relatively fast in a culture without iron. *P. aeruginosa* was chosen for its fast growth rate and biofilm-forming capabilities. *S. lithotrophicus* was selected because it is the closest relative to the FeOB *Gallionella* spp. available in type culture collections.

2.1.2   Culturing media and techniques

2.1.2.1   Culturing of control strains

P. aeruginosa and *S. natans* were maintained in Nutrient Broth (NB) (3 g beef extract, 5 g peptone; per 1000 mL water); Nutrient Agar (NA) was prepared by including 1.5 % agar in NB. *S. lithotrophicus* was maintained in Modified Wolfe (MW)

medium (1.00 g NH_4Cl, 0.20 g $MgSO_4 \cdot 7H_2O$, 0.10 g $CaCl_2 \cdot 2H_2O$, 0.05 g K_2HPO_4, 0.42 g $NaHCO_3$, 5 g FeS; per 1000 mL water) (Emerson and Floyd 2005). Media information is summarized in Table 2.

Control strain liquid cultures were started by preparing tenfold dilutions of stock bacterial suspensions in culture medium. *P. aeruginosa* was incubated for 24 to 48 h; *S. natans* was incubated for five days on average; *S. lithotrophicus* was incubated for one week. All bacteria were incubated at room temperature (approximately 20 °C) without agitation. Culturing on solid media was done by spreading aliquots of liquid cultures in petri dishes containing solid media. Plates were incubated at room temperature for 24 h (*P. aeruginosa*) or one week (*S. natans*, *S. lithotrophicus*). Growth was considered positive if the liquid culture showed visible turbidity or if colonies were apparent on a plate. Freezer stocks (40% final glycerol concentration) were prepared with dense cultures (OD_{600} ~ 1) and stored at -80 °C for future use.

2.1.2.2 Culturing of well FeB

Winogradsky (WI) medium (0.5 g each of $MgSO_4 \cdot 7H_2O$, $NaNO_3$, NH_4NO_3; ferric-ammonium citrate, 0.2 g $CaCl_2$; per 1,000 mL water) (Rodina 1972) and MW medium were used to maintain and enrich FeB from the wells, as well as for evaluation of phage bactericidal effects. A modified MW medium, called Iron Cycling (IC) medium (1.00 g NH_4Cl, 0.20 g $MgSO_4 \cdot 7H_2O$, 0.10 g $CaCl_2 \cdot 2H_2O$, 0.05 g K_2HPO_4, 0.42 g $NaHCO_3$, 0.82 g $NaCH_3COO$, 2.45 g Fe(III) citrate, per 1,000 mL water), was employed for iron metabolism assays. These media also are summarized in Table 2. Given the composition of these media, WI and IC would favor the enrichment of FeRB and subsequently FeOB (once the Fe(III) was reduced to Fe(II) by the FeRB).

FeB liquid cultures were started by preparing tenfold dilutions of the bacterial source (e.g., water from the wells) in culture medium. To better reproduce the ideal growth conditions of FeB, the tubes were filled to 85% capacity, screw-capped and incubated without agitation (static). This ensured that the resulting headspace produced

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the necessary microoxic conditions in the tube (around 0.2 mg/L of dissolved oxygen) (Elliott et al. 2014). Tubes were incubated at room temperature and checked periodically for signs of growth (i.e., turbidity and color changes, particularly the appearance of a yellow/orange hue in MW medium). Freezer stocks (40% final glycerol concentration) were prepared from dense bacterial cultures (OD_{600} ~ 1) and stored at -80 °C for future use.

2.1.2.3   Culturing technique for MW medium

To prepare MW tubes, the indicated (2.1.2.1) amount Ferrous sulfide (FeS) was placed at the bottom of a tube. The tubes were filled to 85% capacity with a solution of 0.15% low-melt agarose prepared in MW. After sterilization, the tubes were supplemented with 1 µL of both ATCC trace minerals® and ATCC vitamins® per mL of medium and carefully bubbled with nitrogen gas for 30 seconds. The tubes were inoculated with well samples by carefully introducing the pipet tip at mid-height and discharging while slowly pulling out the tip. Aliquots for subculturing were taken in the same manner. Incubation was done at room temperature and tubes were not mixed.

In this experimental setup, opposing redox gradients are generated for oxygen and Fe(II). Oxygen diffuses from the top toward the bottom of the tube, and Fe(II) diffuses from the FeS plug at the bottom toward the top of the tube); this prevents abiotic Fe(II) oxidation by keeping Fe(II) from coming into contact with oxygen (Emerson and Floyd 2005) throughout the tube. This, in combination with the lack of organic carbon would exclusively sustain autotrophic FeOB growth (Emerson and Floyd 2005).

2.1.3   Enrichment of well FeB

The first enrichment cycle was started with selected samples of water and solids from the wells. Tubes for subsequent cycles of the enrichment process were inoculated

with aliquots taken from the previous cycle. Each enrichment culture was incubated for one week, and a total of five enrichment cycles were completed. At the end of each cycle, 500 µL were withdrawn from each culture tube, mixed with sterile glycerol to a final glycerol concentration of 40%, and stored at -80 °C for future use.

Table 2. Summary of culture media used in this study. Name, basic composition and uses throughout this work are presented. pH for all media was adjusted to 7±0.1.

Medium	Composition	Use
Iron Cycling (IC)	NH_4Cl, $MgSO_4$, $CaCl_2$, K_2HPO_4, HCO_3^-, Na^+, acetate, citrate, Fe(III)	Iron metabolism assays
Modified Wolfe (MW)	NH_4Cl, $MgSO_4$, $CaCl_2$, K_2HPO_4, HCO_3^-, Fe(II)	Maintenance of *S. lithotrophicus*; well bacteria enrichment and phage assays
Nutrient Broth (NB)	Complex carbon and nitrogen sources	Maintenance of *P. aeruginosa* and *S. natans*; phage assays with *P. aeruginosa*
Winogradsky (WI)	$MgSO_4·7H_2O$, $NaNO_3$, NH_4NO_3, NH_4^+, citrate, Fe(III)	Well bacteria enrichment and phage assays of FeB

2.2 Metagenomics of biofouled wells

2.2.1 Microbial community analysis

Bacterial 16S ribosomal RNA (rRNA) gene sequencing was used to characterize the microbial communities originally present in the well samples as well as those that developed in the enrichment cultures. Bacterial primers 515F (5'-GTGYCAGCMGCCGCGGTA -3') and 909R (5'- CCCCGYCAATTCMTTTRAGT-3') (Hunicke-Smith 2014) were used to amplify approximately 400-base-pair fragments spanning the V4-V5 hypervariable regions of the Bacterial 16S rRNA gene. Sequencing was performed at the Genomic Sequencing and Analysis Facility (GSAF) at the University of Texas – Austin on the Illumina MiSeq platform.

2.2.1.1 DNA extraction and quantification

DNA extraction from well water samples was started by filtering 100 mL of sample through a Power Water DNA® filter (MO BIO Laboratories Inc. Carlsbad, CA). To extract DNA from the enrichment cultures, the cultures were centrifuged at 4,500 xg for 10 min, and the pellet was washed with phosphate buffered saline (PBS) pH 7; the pellet was resuspended in PBS, and the suspension was filtered through a Power Water DNA® filter.

These filters were processed with the Power Water DNA® isolation kit (MO BIO Laboratories Inc. Carlsbad, CA) following instructions from the manufacturer. DNA was quantified using the Quant-iT ™ assay kit (Life Technologies, Inc. Carlsbad, CA) and stored at -20 °C.

2.2.1.2 Sequencing data analysis

Phylogenetic analysis was conducted within the QIIME open-source software pipeline (Caporaso et al. 2010). Forward and reverse reads were stitched, and the raw sequences were trimmed to remove primers and barcodes. Reads were de-multiplexed to assign to each sequence its corresponding sample ID and then quality-filtered using a phred quality score of 99.9 % (q = 30). Operational taxonomic units (OTUs) were clustered at a 97 % identity threshold; chimera sequences were removed with the usearch tool (Edgar 2010); taxonomy was assigned using the Greengenes database (DeSantis et al. 2006). The output from this last operation was exported from QIIME, and genera were assigned a metabolic function according to reported descriptions of these genera (DeSantis et al. 2006; NCBI 2015).

2.3 Obtaining phage suspensions active against FeB

Phages were extracted from activated sludge obtained at the Waller Creek Municipal Wastewater Treatment Plant (Austin, Texas). Activated sludge samples were

collected in sterile polypropylene bottles and processed within 24 h by the methods described in sections 2.3.1 - 2.3.3. Samples were kept at 4 °C at all times.

2.3.1 Direct filtration

Base phage suspensions were obtained according to the protocol described by Tanji et al. (2008). Aliquots of sludge were clarified by centrifugation at 11,100 xg for 5 min. The supernatant was carefully transferred to a new sterile tube, chloroform was added to a final concentration of 1%, and the mix was incubated for 20 min at 4 °C with shaking (120 rpm). This preparation was then filtered through a 0.22-µm polyethersulfone (PES) filter (VWR, Inc. Radnor, PA).

2.3.2 Phage selection in culture media

Additional phage suspensions were developed following the protocol of Belgini et al. (2014). Activated sludge samples were supplemented with an equal volume of MW, NB, WI medium or a 10% powdered iron suspension and incubated at room temperature without agitation for one week.

To lyse the bacteria, chloroform and NaCl were added to final concentrations of 10% and 1 M, respectively. After incubating for 1 h at 4 °C with agitation (50 rpm), the mixture was centrifuged at 4,000 xg for 20 min. The aqueous phase was recovered, and polyethylene glycol 8,000 (PEG-8000) was added to a final concentration of 10% (w/v). The solution was incubated at 4 °C for 24 h and then centrifuged at 11,000 xg for 20 min. The supernatant was discharged and the precipitate was suspended in saline-magnesium (SM) buffer (5.8 g NaCl; 2 g MgSO$_4$·7H$_2$O; 50 mL Tris–HCl pH 7.5 1 M; 5 mL gelatin 2% (w/v); per 1,000 mL water). The suspension was washed with an equal volume of chloroform to remove PEG. The aqueous phase was separated after centrifugation at 4,000 xg for 20 min and used for phage assays.

2.3.3 Enrichment of phages against well samples and control strains

A modification of the protocol presented in Clokie and Kropinski (2009) was used to enrich the populations of the phages of interest. Bacterial cultures were started from freezer stocks by preparing tenfold dilutions in NB or WI (section 2.1.2). After incubating at room temperature without agitation for the proper period (i.e., five days for pure cultures and enrichment cultures of FeB or 24 to 48 h for *P. aeruginosa*) the cultures were inoculated with 1/100 volume of the phage suspension obtained in 2.3.1 and incubated under the same conditions for an additional week. The cultures were then centrifuged at 11,000 x*g* for 10 min; the supernatant was recovered and used to repeat the process. Three enrichment cycles were completed.

2.4 Evaluation of phage suspension activity

The following methods (sections 2.4.1.1 and 2.4.1.2) were employed to test the phage suspensions for their ability to kill the FeB enriched from the well samples. In all cases, equivalent experiments were run using the control strains to verify the effectiveness of the technique. As recommended (Clokie and Kropinski 2009), in these experiments all culture media were used at double strength to promote rapid bacterial growth and supplemented with 2 mM Ca^{2+}, which is required for docking of most phages.

2.4.1 Bactericidal effect of phage suspensions

2.4.1.1 Double layer agar method

Bacterial cultures were started from freezer stocks by preparing tenfold dilutions in WI or MW (section 2.1.2). After incubating at room temperature without agitation for five days, tenfold serial dilutions of phage suspensions were mixed at a 1:1 volume ratio with 0.1 mL of the FeB culture in 3 mL of semi-solid (0.75 % agar added) MW or WI medium (kept liquid at 40 °C until use). The mixtures were then poured over agar plates

of the same culture medium (1.5% agar in desired culture medium, poured on a petri dish and allowed to solidify before use). A plate containing only bacteria served as bacterial growth reference, whereas a plate containing only phage suspension served as a negative control. Plates were incubated at 30 °C until bacterial growth was visible.

2.4.1.2 Liquid culture tests

Following a modification of the protocol described by Fridholm and Everitt, (2005), tenfold serial dilutions of the intended phage suspension were made in SM buffer. Phage dilutions were then mixed in a 1:9 volume ratio with the intended bacterial sample diluted in either WI (for FeB) or NB (for *P. aeruginosa*), and added to individual wells of a microtiter plate. Several dilutions of dense FeB culture (see section 2.1.2) cultures were tested as bacterial inoculum. For *P. aeruginosa*, several dilutions of an overnight culture ($OD_{600} \sim 1$) were tried. Three wells were reserved for experimental controls: one control had only bacterial sample, one control had only phage suspension, and one control had only SM buffer. The total reaction volume was approximately 80% of the well volume. Plates were incubated on the bench, without agitation and at room temperature, and with the top on to prevent contamination. Optical density at 600 nm was recorded at intervals over 48 h, applying the 1-cm path length correction (Synergy® HT, Bio-Tek Instruments, Inc. Winooski, VT). Suspensions were selected for further experiments by their ability to decrease optical density.

2.4.2 Effect of phage suspensions on iron metabolism

2.4.2.1 Test set-up

These experiments were done with batch cultures of planktonic cells in IC medium with purified water (Thermo Scientific, Nanopure #7143. Marietta, OH), so no Fe(II) was initially present in the medium. Bacterial cultures were started from freezer stocks by preparing tenfold dilutions in IC medium (section 2.1.2). After incubating at

room temperature without agitation for five days the desired phage suspensions were added to a final concentration of 0.1% (v/v). Aliquots were taken for iron measurements every five days over the course of four weeks.

2.4.2.2   Iron species measurements

The revised ferrozine method was used for iron speciation monitoring (Viollier et al. 2000). Briefly, the Fe(II)-ferrozine complex is spectrophotometrically (562 nm) quantified on a sample before (A1) and after (A2) a reduction reaction with hydroxylamine. By means of a calibration curve, the concentrations of both Fe(II) and Fe(III) can be determined at the submicromolar level, according to the following equations:

$$C_{Fe(II)} = \frac{A_1 \varepsilon_{Fe(II)} x l \alpha - A_2 \varepsilon_{Fe(III)} x l}{\varepsilon_{Fe(II)} x l \alpha (\varepsilon_{Fe(II)} x l - \varepsilon_{Fe(III)} x l)}$$

$$C_{Fe(III)} = \frac{A_2 - A_1 \alpha}{\alpha (\varepsilon_{Fe(II)} x l - \varepsilon_{Fe(III)} x l)}$$

where $\varepsilon_{Fe(III)}$ and $\varepsilon_{Fe(II)}$ are the molar extinction coefficients for Fe(III) and Fe(II), respectively, l is the optic path length, and α is the dilution factor due to the addition of the reagents. These parameters are derived from the calibration curve. Details are presented in section 6.1.

All solutions for this method were prepared in purified water (Thermo Scientific, Nanopure #7143. Marietta, OH). Absorbance data from the samples was corrected with that of an abiotic control (non-inoculated medium) at each time point. Iron species in the samples were analyzed in terms of excess or deficit with respect to their concentrations in an abiotic control (non-inoculated medium) at the same time point. Ferrozine does not interact with precipitated or complexed iron, so only free dissolved iron species were measured.

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2.5   Statistical analyses

A minimum of three biological replicates (i.e., three separate cultures) were performed for each experiment. Data were tested for homogeneity of variance by means of the Cochran (Cochran 1941) and the Lavene (Brown and Forsythe 1974) tests. Factorial ANOVA was performed to test interactive effects, and One-Way ANOVA was applied when interactions were not significant. To test the bactericidal effect of phages, OD_{600} was used as the variable and sample type (controls vs. well samples) and treatment (phage added or not added) were selected as factors. To test phage effect on iron metabolism, iron species concentrations were used as the variable and sample type (controls vs. well samples), enrichment (original vs. enrichment cycle), enrichment media (MW vs. WI) and treatment (phage added or not added) were specified as factors. All statistical analyses were done using the software Statistica 10 (StatSoft, Inc., Tulsa, OK).

3 RESULTS

3.1 Microbial community of biofouled wells

Analysis of the rRNA gene sequencing data showed that OTUs corresponding to FeB were present in all four well samples, but they were abundant only in well 7 (Troutdale, OR) and well 9 (Fairview, OR), where they comprise about 25% and 5% of the assigned taxa, respectively (Figure 1). Other metabolic groups present include sulfur bacteria, which seem to dominate across all samples, nitrogen bacteria and a few methanotrophs (DeSantis et al. 2006; NCBI 2015). Not shown, but also identified, are several genera commonly found in drinking water, such as *Rhodoplanes, Hydrogenophaga, Rhodococcus, Acinetobacter* and *Bradyrhizobium* (Liao et al. 2013; Pinto, Xi, and Raskin 2012). Bacterial diversity at the taxonomic class level is presented in Figure 15 in the Appendix.
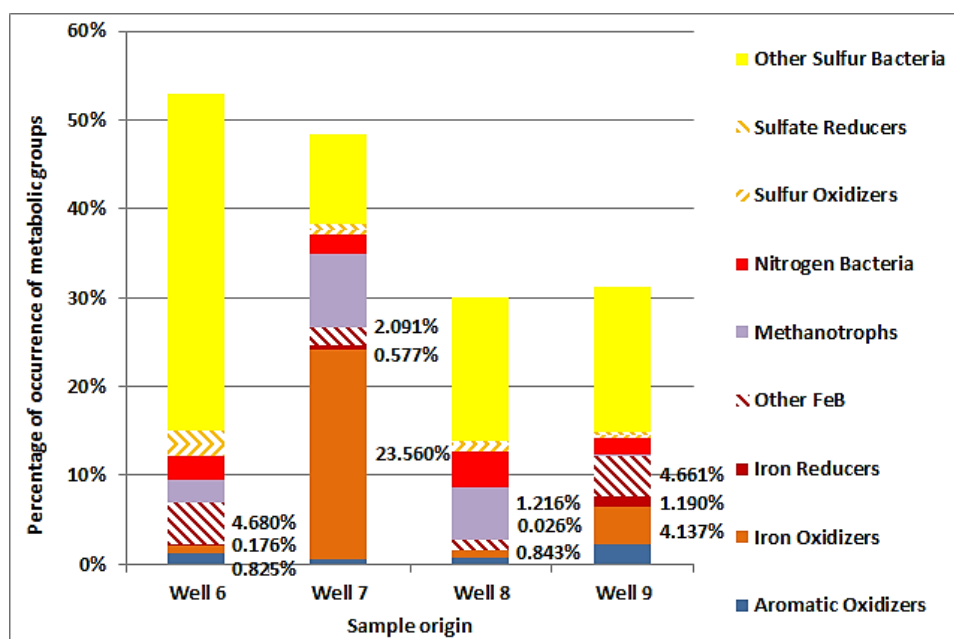
Figure 1. Microbial community composition by metabolic groups. W6, W7, W8 are well samples from Troutdale, OR. W9 is a well sample from Fairview, OR. FeOB (orange), FeRB (dark red) and Other FeB (brown stripes) comprise an important portion of taxa in W7 and W9. Percentages for these groups are shown next to each column. The portion adding up to 100% corresponds to other organoheterotrophs and poorly identified bacteria.

FeB communities for all samples presented similar composition; that is to say, the genera shown in Figure 2 were found in all well water samples, although their abundance differed between samples. In general, *Crenothrix* and *Gallionella* were the most abundant genera within the FeOB group, with *Crenothrix* dominating in samples from Troutdale and *Gallionella* in samples from Fairview (Figure 2). *Geobacter* was the most abundant FeRB, although the FeRB *Geothrix* also was detected in wells 7 and 9 (Figure 2). Some of the OTUs in the "other FeB" group correspond to reads that were not identified through genera but that belong to the order Gallionellales, suggesting that the abundance of *Gallionella*-related species might be even greater.
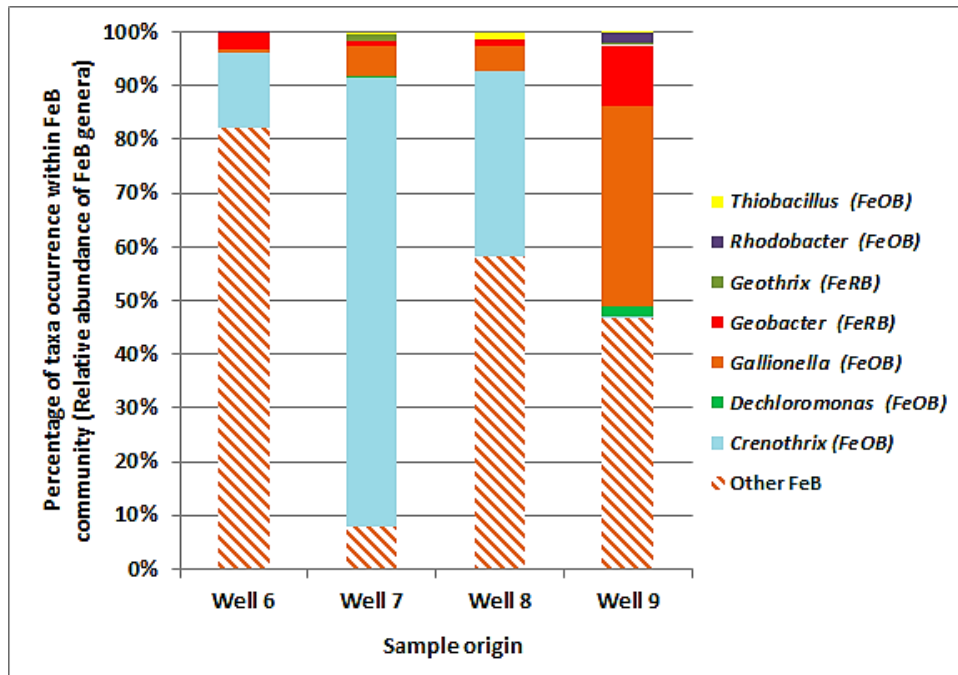
Figure 2. Relative abundance of FeB genera within the FeB community. W6, W7, W8 are well samples from Troutdale. W9-2 is a well sample from Fairview. "Other FeB" includes the FeOB genera *Alicyclobacillus*, the FeRB *Magnetospirillum*, as well as poorly identified Gallionellales and Rhodobaterales.

3.2 Enrichment of FeB

Given the similar FeB communities between all samples (Figure 2), wells 7 and 9 were selected as representative samples for enrichment of FeB and subsequent experiments, to take advantage of their natural FeB abundance (Figure 1). The FeB enrichment was done by serial subculturing in WI and MW media. Five subculturing cycles, numbered 1 through 5, were completed to yield the samples listed in table 3. Unless otherwise specified, aliquots from fifth cycle enrichment cultures carried out in MW (MW-5) and WI (WI-5) were combined in a 1:1 volume ratio and this mixture was used for subsequent experiments. The increase in FeB OTUs due to the enrichment process awaits confirmation by 16S rRNA gene sequencing; however, other phenotypic observations, such as behavior in culture media (Figure 3) suggest that FeB were successfully enriched.

Table 3. Description of enrichment cultures. Nomenclature key for enrichment culture ID: *well number - culture medium - enrichment cycle*.

Original sample	Enrichment medium	Enrichment culture ID	Working samples
Well 7 (W7)	Modified Wolfe (MW) Winogradsky (WI)	W7-MW-1 through W7-MW-5 W7-WI-1 through W7-WI-5	**W7-5**: a 1:1 volume combination of both cycle 5 enrichments
Well 9 (W9)	Modified Wolfe (MW) Winogradsky (WI)	W9-MW-1 through W9-MW-5 W9-WI-1 through W9-WI-5	**W9-5**: a 1:1 volume combination of both cycle 5 enrichments

When cultured in MW, FeB enrichments from well 9 exhibited some of the traits that are used to confirm presence of *Gallionella*, such as a defined yellow or orange band (Figure 3 B) and flake-like growth (Figure 3 C). This was not observed in either the original samples or the FeB enrichments from well 7, where the abundance of *Gallionella* was much lower (Figure 2). Similarly, iron oxidation was not apparent in

MW cultures (i.e., no yellow/orange color developed). Once more, this is presumably because of the lower abundance of *Gallionella* in well 7.
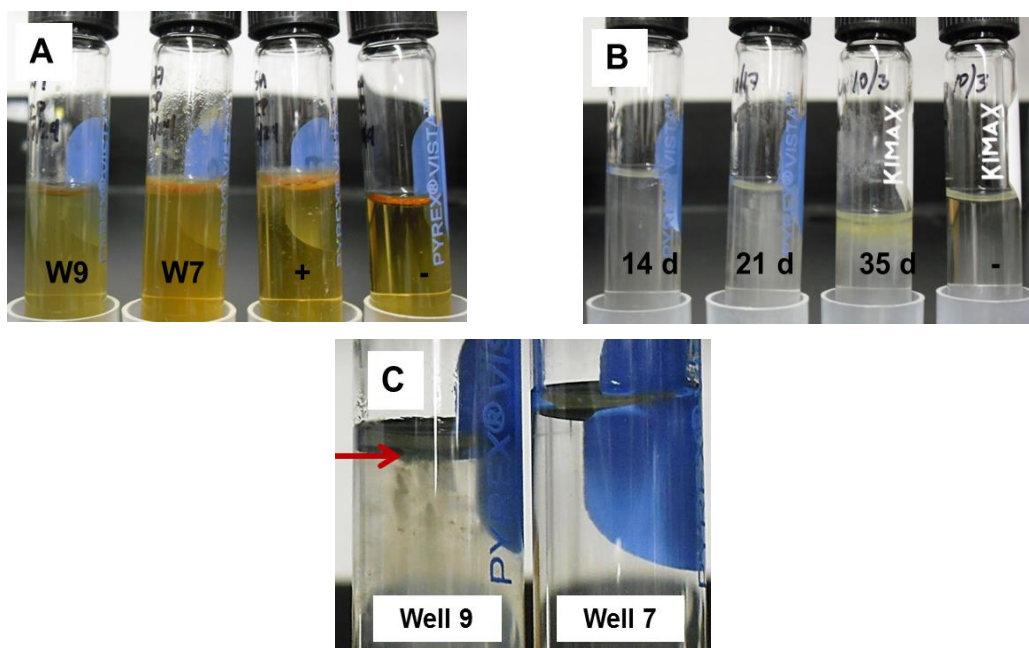
Figure 3. Example of enrichment cultures. A) Samples from well 7 and well 9, *S. natans* (+) and water (-) were inoculated in WI medium. B) Well 9 inoculum growing in MW medium after 14, 21 and 35 days of incubation. A yellow band of increasing intensity corresponds to iron oxidation in the medium inoculated with bacteria but not with water (-). C) Well 7 and 9 samples in MW after 14 days of incubation. The arrow points to presumed *Gallionella*-like flakes appearing in well 9 but not in well 7.

Iron metabolism was analyzed by growing well samples from the first and fifth enrichment cycles and positive/negative controls in double strenght IC, a medium with Fe(II) but no Fe(II) input, and monitoring bulk concentrations of Fe(II) and Fe(III) over the course of four weeks. Results are presented as the difference between the iron concentration in the sample and the negative control (non-inoculated medium) at the same time point. Therefore, a negative value means that a soluble iron species has been removed from the medium relative to the abiotic control, and a positive value indicates that a soluble iron species has been added to the medium relative to the abiotic control.

Both control strains, the iron-oxidizer *S. natans* and the non-iron-bacterium *P. aeruginosa* did not substantially impact the iron speciation. However, culture tubes inoculated with well 7 or well 9 enrichment cultures exhibited similar iron redox cycling to one another. These results are presented in Figure 4; because they behave comparably, *S. natans* and *P. aeruginosa* are combined under "Controls". FeB from wells 7 and 9 are combined under "Well samples" given that they were not statistically different. Note that during the first week they appeared to have consumed Fe(II) and produced Fe(III) and then to have done the opposite during the second through fourth weeks (Figure 4).
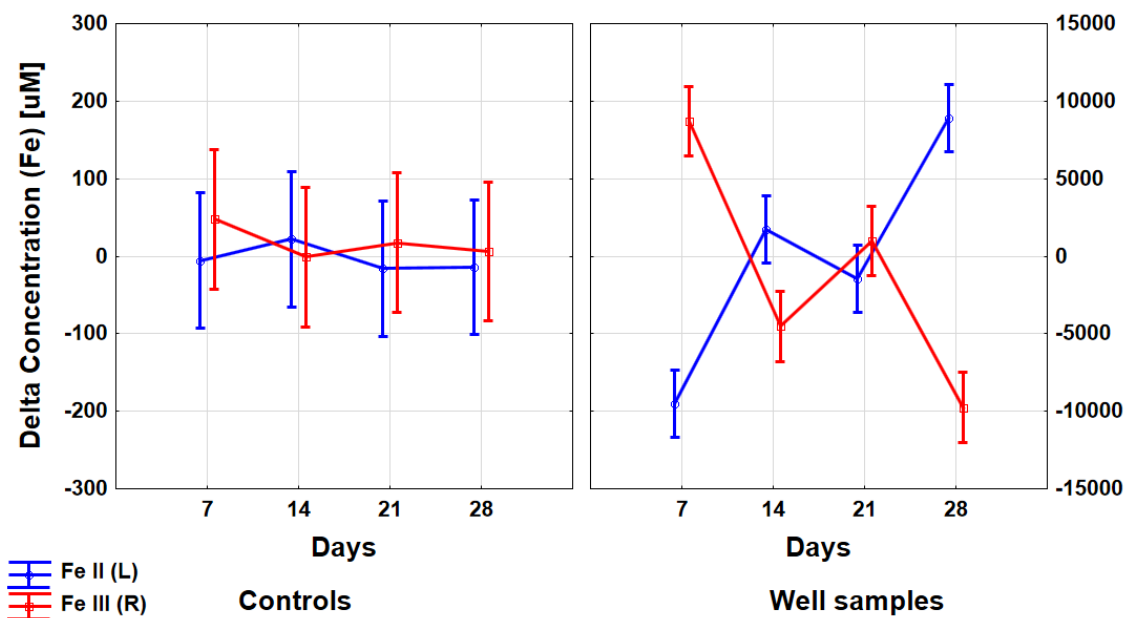
Figure 4. Average iron redox cycling by well samples and control strains in IC medium. Fe(II) concentration in blue and on the left y-axis; Fe(III) concentrations in red and on the right y-axis. Data are presented as the difference in iron concentration between the sample and the non-inoculated (control) medium. Negative values mean that a soluble iron species has been removed from the medium, whereas positive values indicate that a soluble iron species has been added to the medium. Vertical bars denote 95% confidence intervals; p = 0.0001 indicates a significant effect on how the relative concentrations of iron species change in time (days) between controls and well samples.

When evaluating the enrichment process, iron cycling in the first enrichment cycle was found to be similar to that of the last enrichment cycle for both well 7 and well 9 samples (p = 0.9). Combined results for both well samples are shown in Figure 5. This could be implying that the relevant bacterial populations were not lost during subculturing. On the other hand, bacterial cultures enriched in WI performed differently than those enriched in MW (p = 0.03). Figure 6 is representative of the behavior of both well samples, and different amplitudes in iron concentration changes can be observed between bacteria enriched in MW and those enriched in WI. This might suggest that enrichment in different media renders different, although functionally similar (Figure 4), bacterial communities; however, additional data points and microbial community information are needed to confirm this.
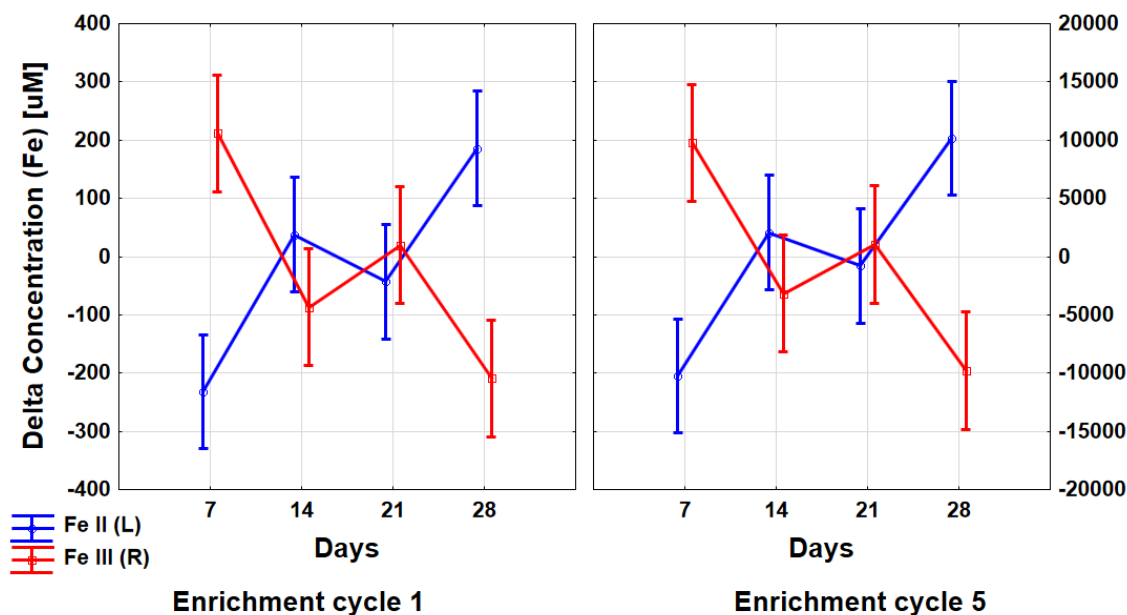
Figure 5. Average iron redox cycling in well samples growing in IC medium as a result of enrichment cycle. Fe(II) concentration in blue and on the left y-axis; Fe(III) concentrations in red and on the right y-axis. Data are presented as the difference in iron concentration between the sample and the non-inoculated (control) medium; vertical bars denote 95% confidence intervals; p = 0.9 indicates similar relative concentrations of iron species between samples from different enrichment cycles.
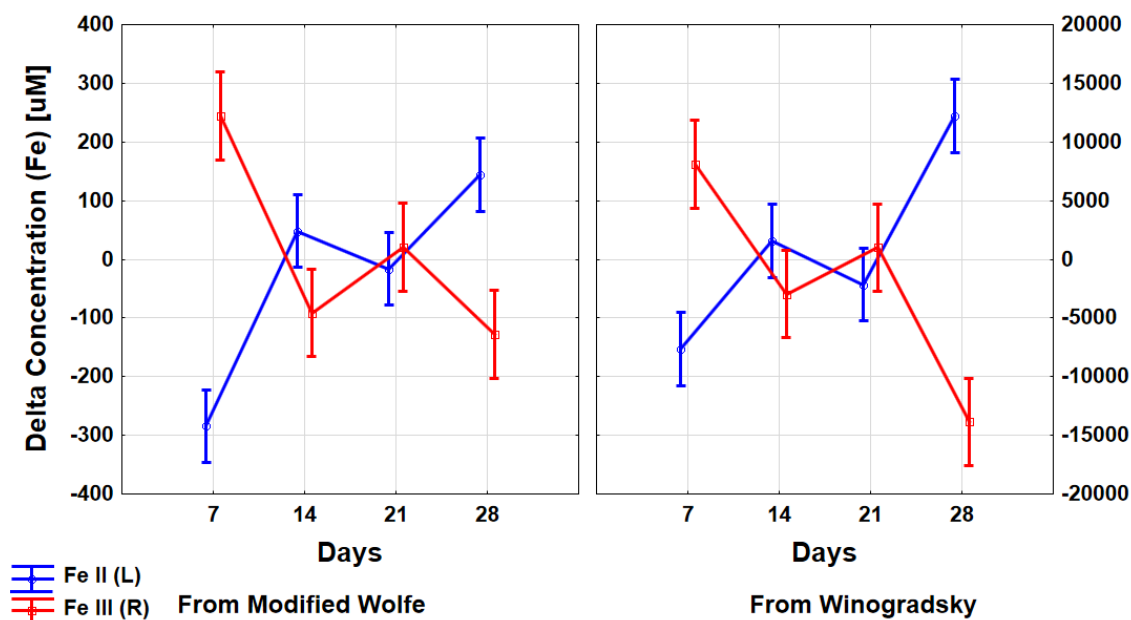
Figure 6. Average iron redox cycling in well samples growing in IC medium as a result of the enrichment culture medium. Fe(II) concentration in blue and on the left y-axis; Fe(III) concentrations in red and on the right y-axis. Data are presented as the difference in iron concentration between the sample and the non-inoculated (control) medium; vertical bars denote 95% confidence intervals; $p = 0.03$ indicates a significant effect on how the relative concentrations of iron species change in time (days) between well samples enriched in MW vs. WI media.

3.3 Phage isolation and evaluation

Several phage suspensions were initially obtained from activated sludge by direct filtration (section 2.3.1), enrichment in culture media that contained bacteria for phage enrichment due to the activated sludge (section 2.3.2) or by enrichment of the filtered activated sludge against fifth cycle enrichment cultures of FeB from the groundwater wells (section 2.3.3) or against control strains cultures (section 2.3.3). Table 4 summarizes the suspensions used in this study.

Double layer agar assays suggested phage activity in some of the suspensions. When *S. lithotrophicus* was challenged with MW-enriched phage suspension (PMW) there was a decrease in the number of colonies developed by *S. lithotrophicus*, as

evidenced by the fewer cotton-like spots visible in the plates in Figure 7. Similarly, when the fifth cycle of well 7 Winogradsky bacterial enrichment culture (W7-WI-5 - Table 3) was inoculated with WI-enriched phage suspension (PWI), fewer colonies could be observed. An example of this is shown in Figure 8, where yellow colonies are more sparse when more phage is added. In both cases, the number of bacterial colonies increased as the amount of added phage suspension decreased.

Table 4. Description of selected phage suspensions obtained. Nomenclature key: except for FAS (filtered activated sludge) the **P** at the beginning of the suspensions ID indicates "Phage" and rest of the letters refer to the culture medium or the bacterial culture in which the phage suspension was enriched.

ID	Name	Description
FAS	Filtered activated sludge	0.2 µm filtered activated sludge (section 2.3.1).
PFe	Iron-enriched phage suspension	Activated sludge incubated one week with powdered iron (section 2.3.2).
PMW	MW-enriched phage suspension	Activated sludge incubated in MW for one week (section 2.3.2).
PNB	NB-enriched phage suspension	Activated sludge incubated in NB for one week (section 2.3.2).
PWI	WI-enriched phage suspension	Activated sludge incubated in WI for one week (section 2.3.2).
PPa	*P. aeruginosa*-enriched phage suspension	FAS enriched against *P. aeruginosa* (section 2.3.3).
PSn	*S. natans*-enriched phage suspension	FAS enriched against *S. natans* (section 2.3.3).
PW7	Well 7-enriched phage suspension	FAS enriched against bacterial enrichment cultures W7-MW-5 + W7-WI-5 (section 2.3.3).
PW9	Well 9-enriched phage suspension	FAS enriched against bacterial enrichment cultures W9-MW-5 + W9-WI-5 (section 2.3.3).
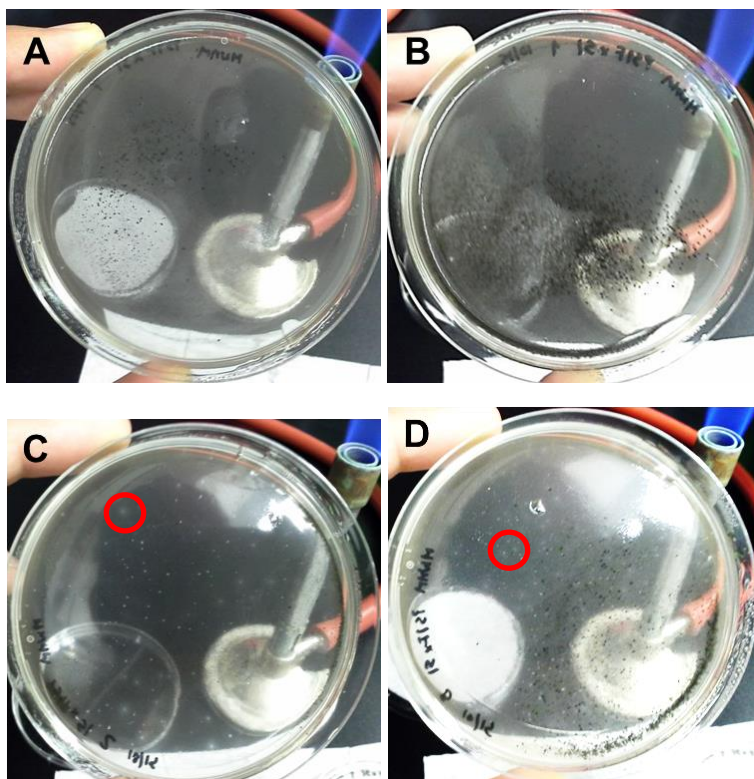
Figure 7. Double layer agar assay with *S. lithotrophicus* (an FeOB) challenged with MW-obtained phage suspension (PMW). A) Only PMW added. B) Bacteria and a ten-fold PMW dilution. C) Bacteria and one hundred-fold PMW dilution. D) Bacteria only. Cotton-like white colonies of *S lithotrophicus* (encircled) are increasingly visible as the amount of phage added decreases.
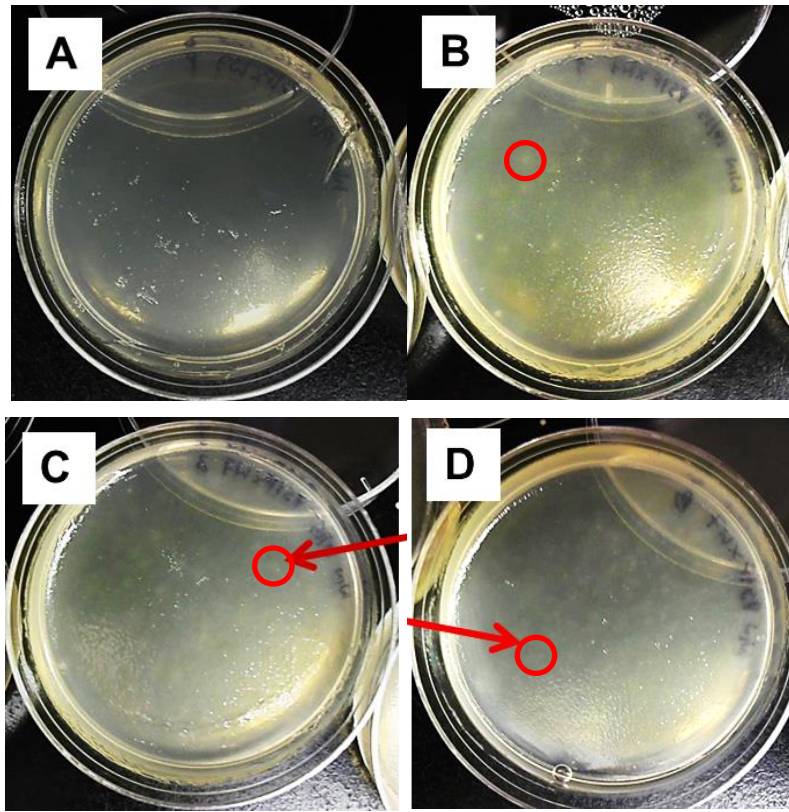
Figure 8. Double layer agar assay with fifth cycle of well 7 Winogradsky enrichment culture (W7-WI-5) challenged with Winogradsky-enriched phage suspension (PWI). A) Only PWI added. B) Bacteria and a ten-fold PWI dilution. C) Bacteria and a hundred-fold PWI dilution. D) Bacteria only. Colonies (encircled) are increasingly visible as the amount of phage added decreases.

Static liquid cultures more accurately reproduce the ideal growth conditions for FeB, because microoxic zones are readily developed. Thus, batch tests were performed to better assess phage activity. The best results were obtained when combining the phage dilutions with a hundred-fold dilution of a dense ($OD_{600} \sim 1$) bacterial culture. The best incubation time for these assays (phage with bacteria) was around 24 h; shorter incubations yielded no significant change in OD_{600} as compared to the initial point, whereas longer incubations were shown to mask the differences between treatments. Presumably due to the mixed nature of the well microbial communities, it was not always possible to produce a consistent initial bacterial inoculum. Even when grown

under the same conditions each time, cultures did not necessarily reach the same optical density within the same incubation period. This fact might have led to the high variability observed (i.e., the large confidence intervals). Therefore, to be able to compare between experiments, results are presented as the fraction of OD_{600} (f- OD_{600}) of the sample with respect to the corresponding bacteria control (no phage addition) for each experiment.

The decrease in relative optical density (f- OD_{600}) indicates that phage in the filtered activated sludge (FAS) proved active against *P. aeruginosa*, *S. natans* and the fifth cycle enrichment of well 9 (W9-5) (Figure 9). *P. aeruginosa* also was affected by PFe phage suspension but not by the rest of the media-enriched phage suspensions. On the other hand, W7-5 seemed to be unaffected by all of the phage suspensions as tested by this assay because f-OD_{600} was not different from the control (no phage addition) (Figure 9). When comparing the overall difference in f-OD_{600} between phage-treated and untreated samples, there was an overall significant effect of the phage addition (p = 0.001) on the turbidity of the bacterial cultures. However, when comparing the results grouped by dilution factor, no substantial differences were observed (i.e., the 1:10 phage dilution was not more effective against the bacterial culture as compared to the 1:100 phage dilution). This might be due to the fact that OD_{600} approaches the value of the control (no phage addition) at higher phage dilutions (recovery of f-OD_{600}). Figure 10 shows an example of this observation.
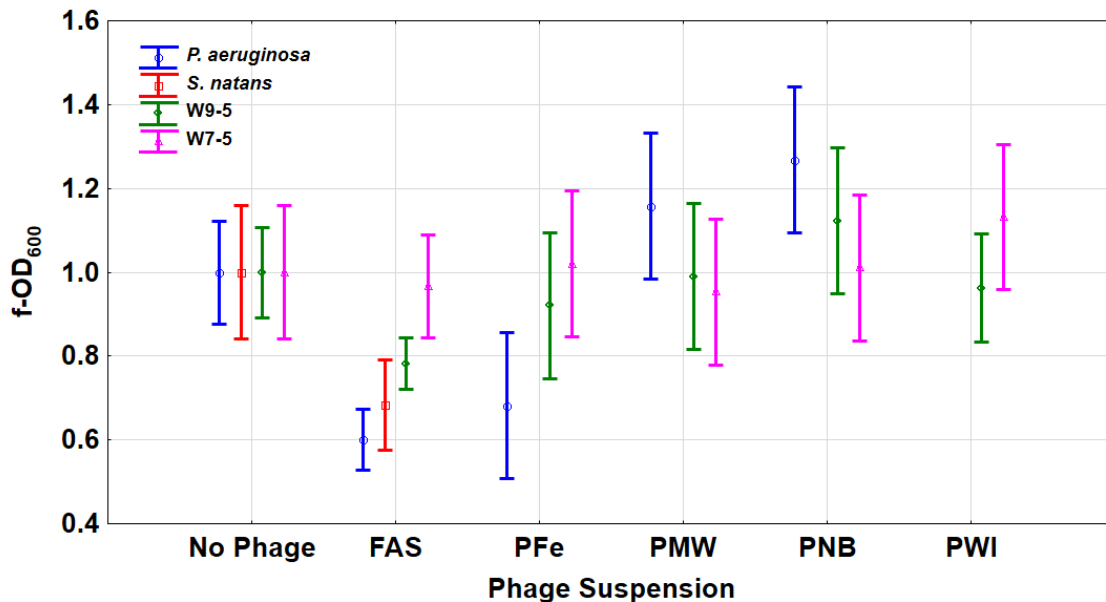
Figure 9. Performance of selected phage suspensions against well samples and control strains. Results correspond to a hundred-fold dilution of each phage suspension. Data are presented as the fraction of remaining optical density at 600 nm (f-OD$_{600}$) relative to the untreated sample (No Phage); vertical bars denote 95% confidence intervals; overall effect of phage treatment (versus no phage treatment) was significant at p = 0.0001.
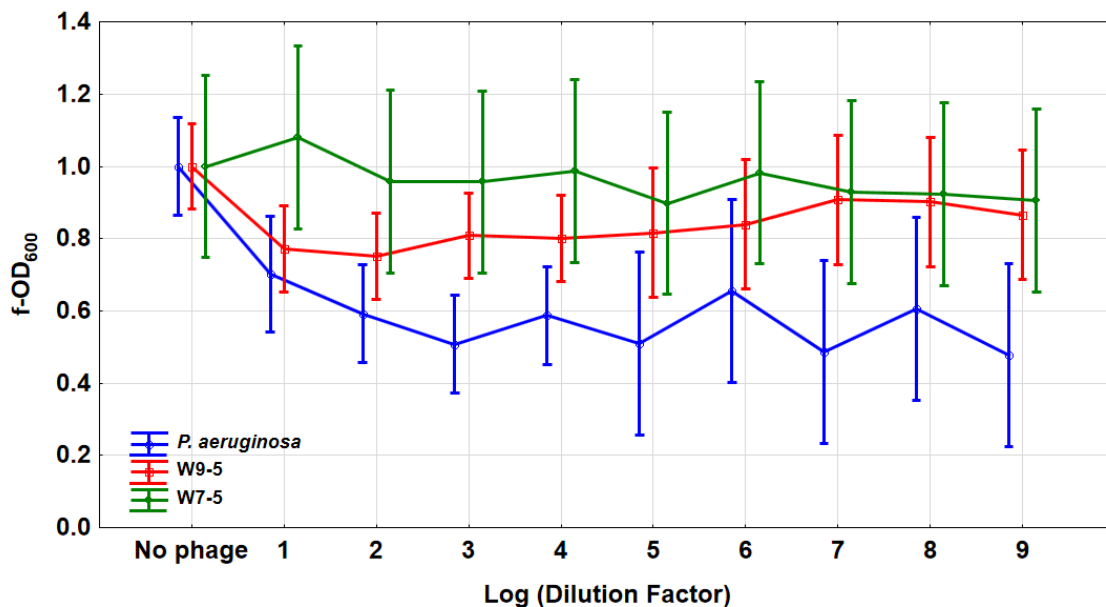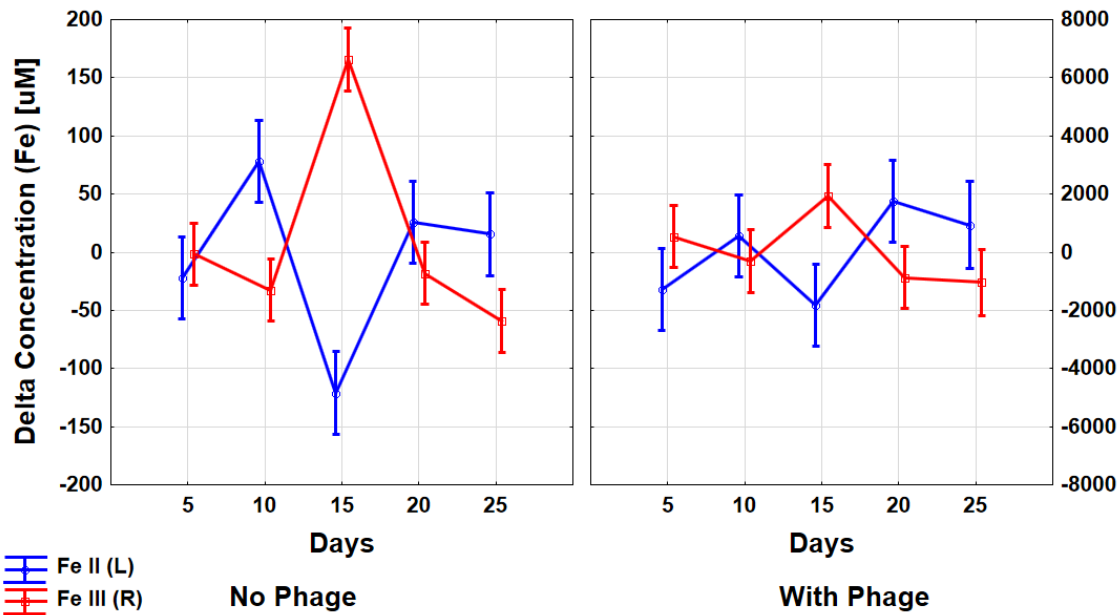

Figure 10. Effect of phage treatment with FAS grouped by dilution factor. Data are presented as the fraction of remaining optical density at 600 nm (f-OD$_{600}$) relative to the untreated sample (no phage); vertical bars denote 95% confidence intervals.

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The effect of phage treatment on bacterial iron cycling was then assessed using double strength Iron Cycling (IC) medium, which contains Fe(III) but not Fe(II). Tubes containing IC medium were inoculated with original well samples, cycle five enrichment cultures samples, and control strains. The bacterial cultures were challenged with phage suspensions developed against each bacterial sample: PPa, PSn, PW7 and PW9 (Table 4), and Fe(II) and Fe(III) concentrations were measured periodically over four weeks.

When comparing the relative iron species concentrations of well samples upon phage treatment, decreased amplitude in the iron cycling was evident, particularly at 10 and 15 days of incubation (Figure 11). Statistical analysis showed a significant effect of phage treatment on how the relative concentration of iron species changed during the experiment ($p < 0.00001$).

In agreement with previous observations (Figure 5), iron cycling was no different between initial and final stages of enrichment (Figure 12 ; $p = 0.28$). It was, however, different between treatments (i.e., phage vs. no phage) for both well samples (Figure 12; $p < 0.05$). Also as expected, the relative concentrations of iron species remained similar for both control strains, regardless of the treatment (Figure 13; $p = 0.69$). As expected, both the non-FeB *P. aeruginosa* and the FeOB *S. natans* were seemingly unable to cycle iron in IC medium (containing Fe(III)), and this did not change when phages were added to the culture.

Figure 11. Overall effect of phage treatment on iron redox cycling in well samples growing in IC medium. Phage suspensions PW7 and PW9 were tested against well 7 and well 9 samples, respectively Results are combined under "With Phage" panel. "No Phage" panel shows combined results non-treated well 7 and well 9 samples. Fe(II) concentration in blue and on the left y-axis; Fe(III) concentrations in red and on the right y-axis. Data are presented as the difference in iron concentration between the sample and the non-inoculated (control) medium; vertical bars denote 95% confidence intervals; $p = 0.0001$ indicates significant interactive effect between time (days) and treatment (with phage vs. no phage) on the relative concentrations of iron species.
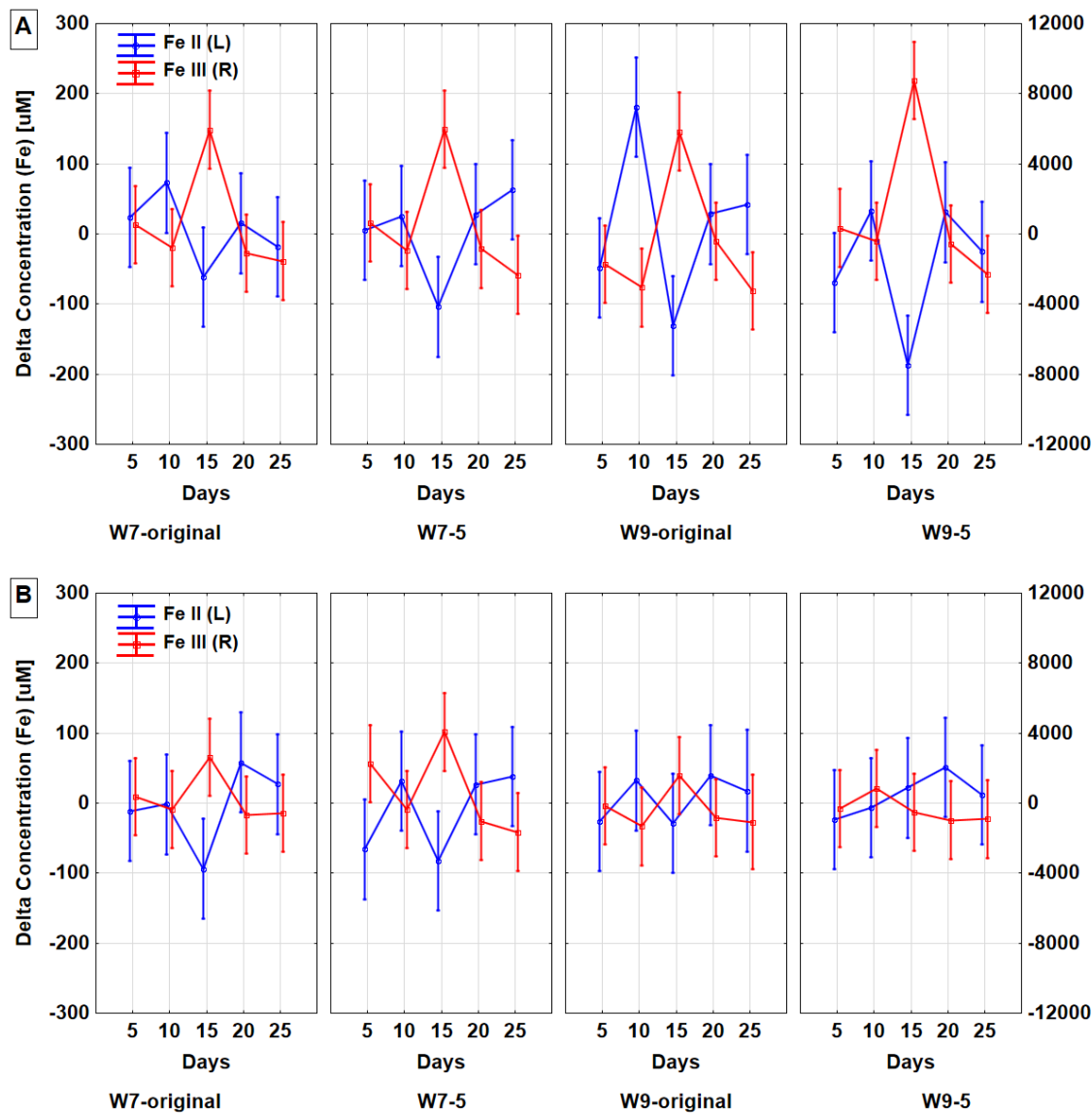
Figure 12. Effect of phage treatment on iron redox cycling of individual well samples growing in IC medium. A) No phage added. B) W7-enriched phage suspension (PW7) was added to well 7 samples and W9-enriched phage suspension (PW9) to well 9 samples. Fe(II) concentration in blue and on the left y-axis; Fe(III) concentrations in red and on the right y-axis. Data are presented as the difference in iron concentration between the sample and the non-inoculated (control) medium; vertical bars denote 95% confidence intervals. Relative concentrations of iron species change significantly because of interactive effect between time (days) and treatment (with phage vs. no phage) for each well sample ($p < 0.05$), but not between well samples or enrichment cycles.
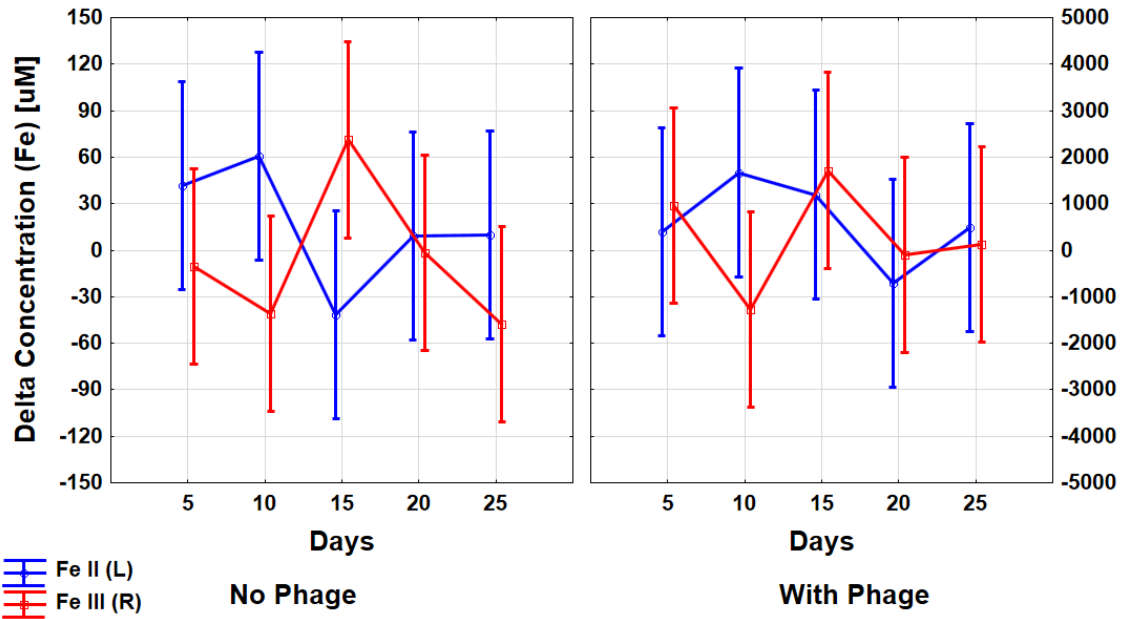
Figure 13. Relative concentration of iron species in cultures of the control strains in IC medium with and without phage treatment. *S. natans*-enriched phage suspensions (PSn) and *P. aeruginosa*-enriched phage suspension (PPa) were tested against control strains *S. natans* and *P. aeruginosa*, respectively. Fe(II) concentration in blue and on the left y-axis; Fe(III) concentrations in red and on the right y-axis. Data are presented as the difference in iron concentration between the sample and the non-inoculated (control) medium; vertical bars denote 95% confidence intervals; no significant change in relative concentration of iron species between treatments (phage vs. no phage).

4 DISCUSSION

Given their specificity, their ability to propagate and their confinement to the prokaryotes (Adams 1959), bacteriophages constitute a promising tool for the control of unwanted bacteria in a diverse array of situations. But before they can be exploited in this capacity, they must first be found, characterized, and their performance evaluated. The purpose of this study was to assess the applicability of a phage therapy to control bacterial consortia responsible for iron-mediated biofouling in water supply wells. To achieve this objective, an appropriate target had to be identified and a suitable evaluation method developed. In other words, it was necessary to characterize the biofouling bacteria and to select a trait to monitor during treatments. The challenge was to accomplish this while working with a mixed community representative of natural microbial diversity.

4.1 Identity of well biofouling microbial consortia

Published studies assessing the microbial community of drinking water sources and systems rarely go deeper than the taxonomic family level. Nonetheless, the microbial communities of the wells analyzed in this study are similar to those reported for equivalent systems (Gault et al. 2012; Kato et al. 2013; Navarro-Noya et al. 2013; Quaiser et al. 2014). Some of the more frequently reported neutrophilic FeOB were present in these samples, as sequences matching the genera *Gallionella*, *Thiobacillus*, *Rhodobacter* were identified. However, *Leptothrix*, a commonly found FeOB, was not detected in the current study. This might be due to the fact that *Leptothrix* is not listed in the Greengenes database (DeSantis et al. 2006) used in this study; furthermore, *Leptothrix* has been historically in close taxonomic association with *S. natans* (Mulder and van Veen 1963) and *Crenothrix* (Jackson 1902), and authors seem to indistinctly assign the label "*Leptothrix*" or "*Crenothrix*" based on morphology rather than genetics.

Information about the ecological niches of the taxa identified here also is lacking. Methane and FeB have been reported to appear in close physical association (Kato et al. 2013; Quaiser et al. 2014) although a possible ecological relationship between the two has not yet been elucidated. At low pH, sulfur-bacteria also co-occur with acidophilic FeOB (Baker and Banfield 2003), but given the acid production intrinsic to sulfur metabolism, sulfur-bacteria have been proposed to compete with neutrophilic FeOB, (Chapelle and Lovley 1992; Gault et al. 2012). Denitrifiers, also detected, might be involved in nitrate-dependent iron oxidation, a process in which bacteria use Fe(II) as electron donor and nitrate as electron acceptor (Carlson et al. 2012). This would contribute to the Fe(II) oxidation without the involvement of classic aerobic FeOB. Although denitrifiers were not as abundant as other groups (Figure 1), their contribution to the accumulation of BIOs should not be dismissed. In any event, the actual ecological roles of these groups of microorganisms have not been well studied and remain largely uncharacterized.

4.2   Iron cycling as a surrogate for community function

Contrary to the co-occurrence of FeB with other metabolic groups, the co-occurrence of FeRB and FeOB has been more thoroughly addressed. These two groups of FeB have been reported in both natural (Blöthe and Roden 2009; Roden 2012; Kato et al. 2013) and engineered (Wang et al. 2014) environments. Using artificially constructed consortia, FeOB and FeRB were proven to be able to engage in a microbial mediated iron redox cycling, where each group maintains the substrate supply for the other (Roden et al. 2004). This was later confirmed with naturally occurring communities, on the basis of both taxonomic and mineral composition of the resulting iron precipitates (Elliott et al. 2014). Additionally, there might be a role for aerobic heterotrophs because they could control the concentration of dissolved oxygen, effectively creating microoxic (2 - 10 % O_2) zones where FeOB can thrive (Elliott et al. 2014).

No reports of iron cycling monitoring were found in the context of phage treatment or biofouling in general. Nevertheless, the results obtained in his study suggest that this redox cycling of iron might constitute the perfect trait to monitor community function because it depends on community composition and is directly related to the problem that is being addressed.

Biological function has traditionally been addressed by monitoring a trait in reciprocal transplant experiments, in which the organism of interest is introduced into a different environment and the original environment is also modified (Reed and Martiny 2007). By monitoring a trait of interest it is possible to discern whether the environment, the community, the individual or combinations of these factors, are necessary for the trait to manifest. While there are inherent problems to manipulating microbial communities in the field (Peterson, Allen, and Holling 1997), it is possible to carry on equivalent experiments in a laboratory setting. One example is subjecting different communities to different media and testing for compositional or functional convergence, which is essentially what was done in this study. The enrichment culture media used were arguably diametric opposites; samples grown in MW were provided Fe (II) as sole electron donor , whereas those cultured in WI profited from a more labile carbon source (citrate) and Fe (III). In principle, MW was meant to exclusively support to growth of FeOB while WI could favor both FeB. Yet, the resulting bacterial communities kept their iron cycling capacity throughout (Figure 5, Figure 6). According to these results, the selection pressures did not exclude the survival of either group of FeB, and thus the iron cycling capacity was maintained.

4.3   Enrichment of mixed phage suspensions against FeB

Phage suspensions also had to reflect the diversity of the samples, in that they had to contain phages active against the relevant bacterial populations (i.e., FeOB and FeRB). Previous research has addressed the use of mixed phage suspensions to control mixed bacterial communities (Sillankorva 2008), but these phage suspensions were

artificially produced by combining two previously isolated phages. To the best of our knowledge, this is the first attempt at using naturally occurring mixed phage suspensions and at evaluating phage activity through iron redox cycling. However this approach was not without problems.

The traditional route for phage isolation and evaluation almost invariably includes the selection of phages of interest through plaque development in agar plates. The double layer agar method relies on the ability of the target bacterium to form a complete lawn on a solid medium, and on the time-sensitive localized expansion of plaques due to phage infection (Clokie and Kropinski 2009); if results are read too early (plaque formation not visible yet) or too late (plaques might be covered by bacterial regrowth), they are prone to be falsely interpreted as negative. However, bacteria enriched from the wells proved to be very slow-growers, requiring incubations periods of at least one week to form colonies on a plate. Moreover, organisms like *Gallionella* , one of the dominant iron-oxidizers identified in well 7 (Figure 2), do not even grow on solid medium (Emerson and Floyd 2005), therefore the double layer agar results obtained for well 7 (Figure 7) might be biased towards what is able to grow under the assay conditions and not necessarily reflect activity against the intended target. Thus, alternative methods for obtaining and evaluating phages of interest were pursued.

Phages were selected against mixed cultures instead of pure cultures. Enrichment in culture media (Modified Wolfe, Nutrient Broth, Winogradsky; Table 4), was intended to boost the number of phages active against bacteria that share environmental requirements with those from the wells (Belgini et al. 2014). Enrichment in the presence of target bacterial populations was aimed to specifically increase the titer of phages active against that particular sample (Clokie and Kropinski 2009). Phage enrichment in culture media might be producing different mixtures of phages, as the same bacterial samples seemed to respond differently to them (Figure 9). Phage enrichments against target bacteria populations were selected for downstream analysis.

A microtiter plate assay was adapted from Fridholm and Everitt (2005) to evaluate phage activity. One of the challenges on this work was the high variability in the output optical density that led to cancelling out the effect of the dilution factor (Figure 10) and therefore to the inability to titrate the suspensions. Fridholm and Everitt (2005) found that the microtiter plate phage assay is sensitive to both the bacterial input and the incubation time, just as the plaque method is. So one possible explanation to the problem evidenced in this work is that the timing is not right. However, other incubation times did not provide meaningful information (section 3.3).

Bacteria-phage dynamics such as predator-prey interactions (Julia et al. 2014; Harper et al. 2014; Heilmann, Sneppen, and Krishna 2012) could also play a role. In this case the surge and decline of populations as the result of predation or competition might result in different ratios of phage and bacteria being captured (measured) at different times and different dilutions. In relation to this, it is possible that the phages are selectively eliminating some bacteria (e.g., FeB) lifting the competitive pressure placed over other groups (e.g., aerobic heterotrophs), which could keep contributing to the turbidity observed. In support of this idea is the fact that *P. aeruginosa*, a pure culture, appeared to be more sensitive to the different phage suspensions than were the well bacteria communities (Figure 9). More quantitative assays, such as qPCR, might be needed to elucidate this issue.

5   CONCLUDING REMARKS

Microbial communities from well samples were interrogated using a metagenomic approach. Several FeOB and FeRB, including *Gallionella*, *Crenothrix* and *Geobacter*, were identified, along with sulfur-bacteria, nitrogen-bacteria and methane-bacteria. FeB were found to be abundant only in two of the groundwater wells studied, i.e., well 7 and well 9. These well samples were used for enrichment of both iron-oxidizers and iron-reducers and subsequently for the selection of phages active against these bacterial communities. Iron metabolism assays showed that the enriched iron-bacteria cultures were capable of engaging in an iron redox cycling under laboratory conditions, whereas control strains (*S. natans*, *P. aeruginosa*) were not. Furthermore, this ability was shown to be impaired upon phage treatment, a fact that, when combined with optical density assays, indicate relevant phage activity.

These results suggest that iron redox cycling could be happening in the natural habitats of these bacteria. Biofilm formers identified in these wells such as the microbial aggregator *Acinetobacter* (Simões, Simões, and Vieira 2008), could be taking up the role of aerobic regulators of oxygen concentration proposed by Elliott et al. (2014). When put together, this seems to be of importance regarding iron-related well biofouling, and judging by the results obtained in this study, the use of bacteriophages to address this problem is a possibility.

Future work may need to confirm that these bacterial communities of FeOB and FeRB are able to cycle iron between them and support each other in this way. For this, iron cycling experiments should be repeated in a medium containing only Fe(II), and soluble and insoluble iron should be measured. Thus, a solubilization step would need to be included in the iron measurement protocol. To further address the idea of iron redox cycling occurring in the wells, equivalent experiments with fully developed biofilms should be conducted. In this regard, annular reactors have been set up with Iron Cycling medium to grow biofilms from original and enriched samples from wells 7 and 9; both microbial community composition and iron speciation are being monitored for

phage-challenged and unchallenged biofilms. Finally the specificity of the phage suspensions must be characterized by cross-testing each suspensions against all other bacterial samples, and possibly pure cultures of other bacteria.

6 APPENDIX

6.1 The ferrozine method (Viollier et al. 2000)

The ferrozine method was used for iron speciation monitoring. All solutions for this method were prepared in nanopure water (Thermo Scientific, Nanopure #7143. Marietta, OH).

6.1.1 Reagents

A. Ferrozine: 10 mM in 100 mM ammonium acetate (CH_3COONH_4).

B. Reducing agent - hydroxylamine hydrochloride ($H_2NOH \cdot HCl$): 1.4 M, prepared in 2 M analytical grade hydrochloric acid.

C. Buffer - ammonium acetate: 10 M, adjusted to pH 9.5 with ammonium hydroxide (NH_4OH).

Standards were prepared in nanopure water from a 1000 mg/mL Fe(III) stock solution (17.86 mM of $FeCl_3$ in 10 mM HCl)). A nanpure water sample was used as blank.

6.1.2 Procedure

i. One part of reagent A is mixed with 10 parts of the sample or standard. A_1 is recorded as the absorbance at 562 nm.

ii. Reduction step: one part of reagent B is added to 5 parts of the solution resulting from (i). This solution is allowed to react for 10 min to complete the reduction of Fe(III).

iii. One part of reagent C is added to 20 parts of the solution obtained in (ii), and A_2 is recorded as the absorbance at 562 nm.

iv. Steps (2) and (3) are repeated just for the standards and A_2' is measured, again, as absorbance at 562 nm.

6.1.3 Analysis

The absorbance of the blank was subtracted from that of each standard and the corrected A_2 and A_1 datasets were plotted against Fe^{+3} concentration. Regression lines were forced through zero, and their slopes were determined. Fe(II) and Fe(III) were calculated according to the following equations:

$$C_{Fe(II)} = \frac{A_1 \varepsilon_{Fe(II)} x l\alpha - A_2 \varepsilon_{Fe(III)} x l}{\varepsilon_{Fe(II)} x l\alpha(\varepsilon_{Fe(II)} x l - \varepsilon_{Fe(III)} x l)}$$

$$C_{Fe(III)} = \frac{A_2 - A_1\alpha}{\alpha(\varepsilon_{Fe(II)} x l - \varepsilon_{Fe(III)} x l)}$$

where $\varepsilon_{Fe(II)} l$ and $\varepsilon_{Fe(III)} l\alpha$ are the slopes of the regression lines for the A_1 (Figure 14 A) and A_2 (Figure 14 B) standards datasets, respectively. The dilution factor α is the proportionality coefficient between A_2' and A_2 (Figure 14 C). Iron species were then analyzed in terms of excess or deficit with respect to their concentrations in the abiotic control (non-inoculated medium) at the same time point.

A
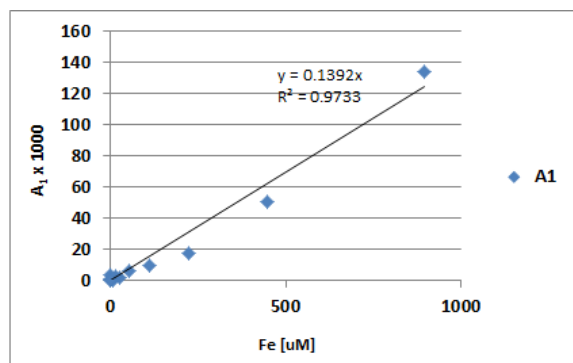
B
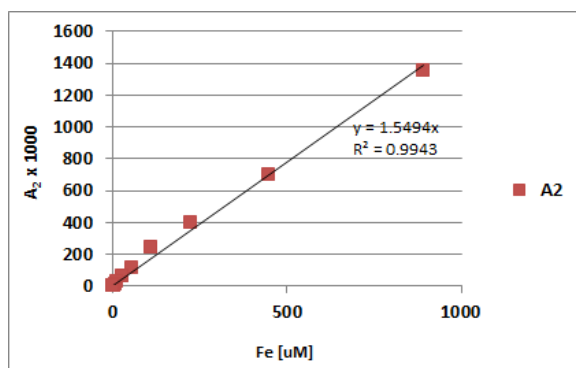
C
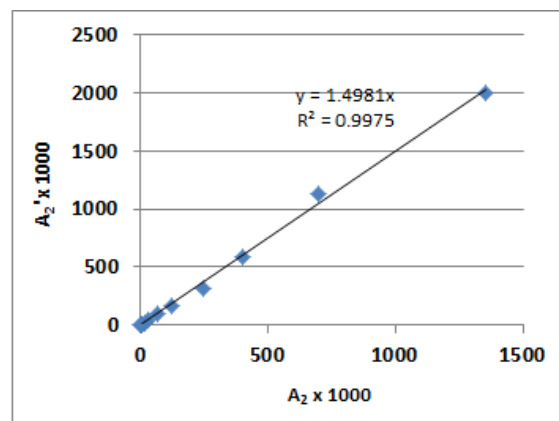
Figure 14. Example of calibration curves for the ferrozine method. A: A_1 is plotted against concentration of standards, and $\varepsilon_{Fe(II)}l$ **is estimated as the slope of this line**. . **B:** A_2 is plotted against concentration of standards, and $\varepsilon_{Fe(III)}l\alpha$ **is estimated as the slope of this line**. **C:** A_2' is plotted against A_2 and α **is calculated as the slope of this line**.

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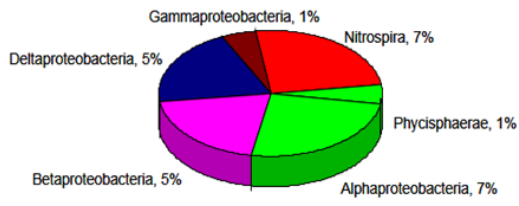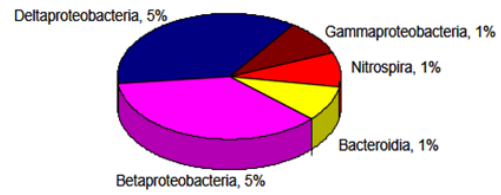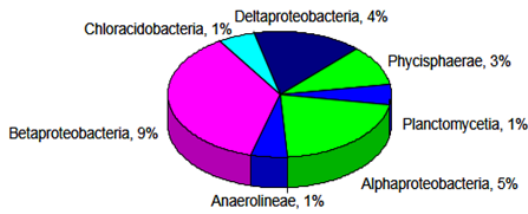
Figure 15. Bacterial diversity of well samples at taxonomic Class level. Classes with more than 1 % of occurrence are shown for well samples from Troutdale, OR (6, 7 and 8) and Fairview, OR (9).

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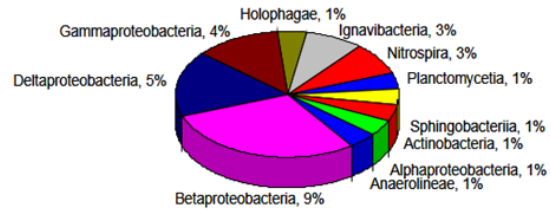
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