Electronic Supplementary Material (ESI) for Environmental Science: Water Research & Technology.

Electronic Supplementary Information

for

Bacteriophage inactivation as a function of ferrous iron oxidation

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The Electronic Supplementary Information consists of 9 pages, including 6 sections, 6 figures, and 4 tables:

1. Verification of phage recovery using the beef broth elution method
2. Calculation of apparent k values
3. R script for linear regression analyses
4. Summary of regression models
5. Floc formation at varying ferrous iron doses
6. Impact of ferrous iron oxidation on floc formation

ESI 1: Verification of phage recovery using the beef broth elution method

Virus recovery was compared after coagulation with ferric chloride and ferrous chloride (8.5 mg/L Fe). After coagulation, bacteriophage concentration was compared in filtered (0.45 µm PTFE filter) water (indicating the sum of physical removal and inactivation) and in a sample eluted with an equal volume of 6% beef broth (pH 9.5) (indicting the degree of inactivation). As shown in Figure ESI.1, the majority of the spiked MS2 and P22 (~10^7 PFU/mL) were recovered by beef broth elution after ferric chloride coagulation, demonstrating the validity of this recovery method. However, no recovery was observed in the ferrous chloride test, demonstrating virus inactivation.

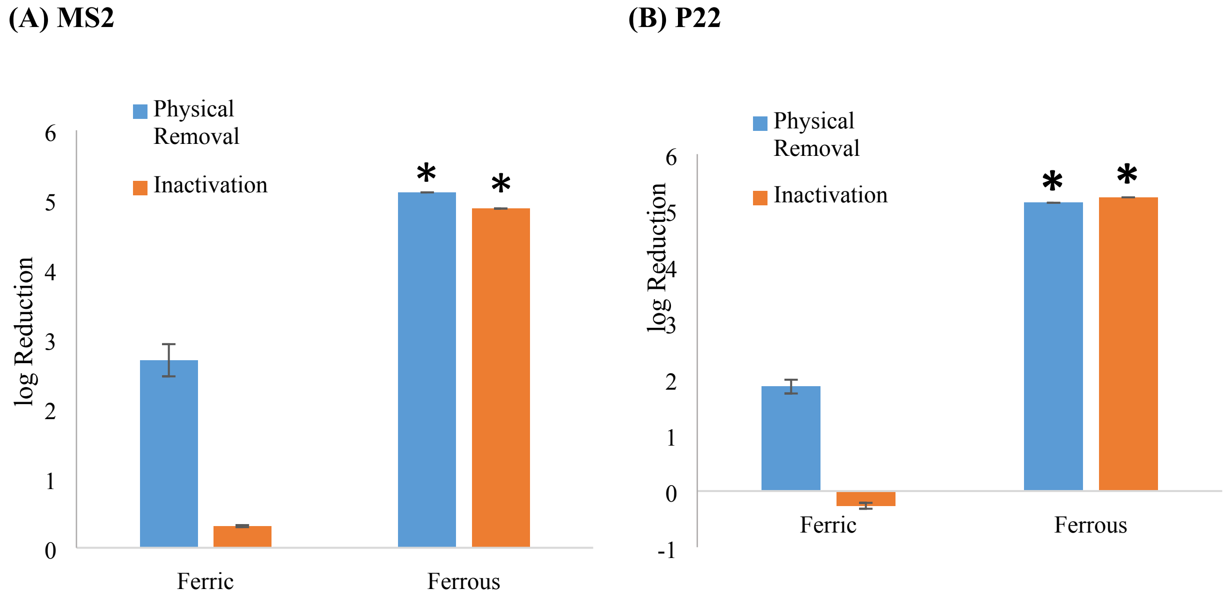

Figure ESI.1: Confirmation of the beef broth elution method using ferric and ferrous chloride (8.5 mg/L Fe) for bacteriophages (A) MS2, and (B) P22. Error bars represent ±1 standard error of triplicate experiments. Asterisks (*) indicate removal beyond the detection limit (plaques were too few to quantify at the lowest dilution, and are shown at the limit of detection).

ESI 2. Calculation of apparent k values

Change in ferrous concentration over time was measured after addition of $FeCl_2$ (2.5 mg/L Fe) to 3 mM bicarbonate solution over varying pH (pH 5.99 to 8.06, Table ESI.1) and dissolved oxygen (0.47 to 4.77 mg/L O_2, Table ESI.2) conditions. Tests of varying pH were conducted with constant dissolved oxygen (8.5 ± 0.2 mg/L DO); tests of varying dissolved oxygen were conducted at constant pH (6.92 ± 0.07). The interpolated surfaces of these ferrous oxidation curves are shown in Figure ESI.2. Exponential trendlines for each condition were fitted in Excel to derive a formula of the format:

$$C_{ferrous} = C_0 \exp[-kt] \tag{ESI 1}$$

The rate constant, k, was determined to be a function of the concentrations of hydroxide and dissolved oxygen, as shown in Figure 1 of the main text.

Table ESI.1: Calculated k values based on iron oxidation rates from pH 6 to 8.

pH	$[OH^-]$ (M)	$[O_2]$ (M)	k (min^{-1})
5.99	9.77E-09	2.71E-04	0.005
6.31	2.04E-08	2.61E-04	0.004
6.46	2.88E-08	2.71E-04	0.014
6.54	3.47E-08	2.61E-04	0.006
6.82	6.61E-08	2.61E-04	0.027
6.96	9.12E-08	2.71E-04	0.116
7.2	1.58E-07	2.61E-04	0.115
7.66	4.57E-07	2.71E-04	0.327
7.75	5.62E-07	2.61E-04	0.451
7.96	9.12E-07	2.61E-04	0.821
8.06	1.15E-06	2.61E-04	1.24

Table ESI.2: Calculated k values based on iron oxidation rates from 0.5 to 4.8 mg/L dissolved oxygen.

Dissolved Oxygen (mg/L)	pH	$[OH^-]$ (M)	k (min^{-1})
0.47	6.97	9.33E-08	0.015
0.9	6.94	8.71E-08	0.008
0.94	7.04	1.1E-07	0.035
1.63	6.9	7.94E-08	0.03
2.24	6.89	7.76E-08	0.036
2.5	6.95	8.91E-08	0.064
3.03	6.84	6.92E-08	0.057
4.77	6.83	6.76E-08	0.076

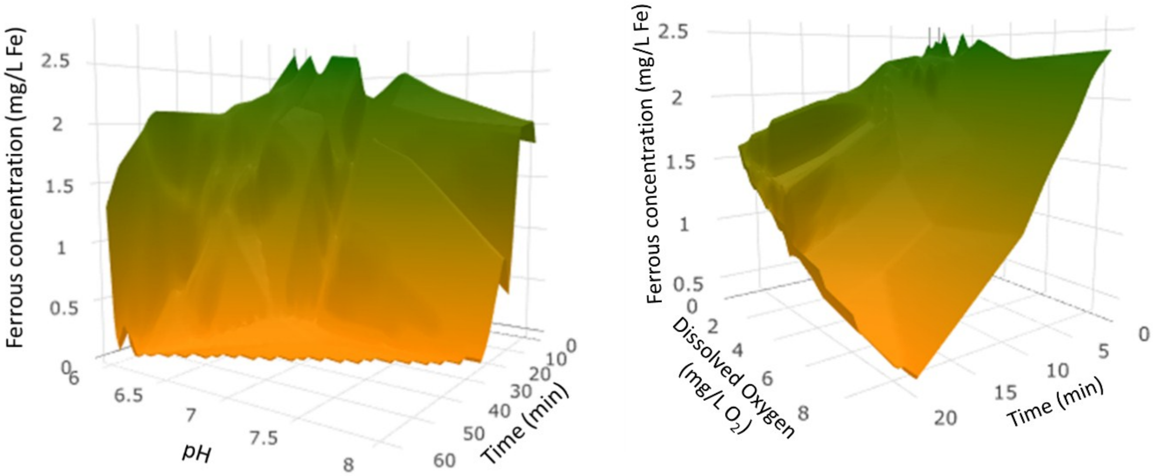
(A) Hydroxide concentration / pH **(B) Dissolved oxygen concentration**

Figure ESI.2: The impact of (A) pH and (B) dissolved oxygen on ferrous oxidation rates. Surfaces were interpolated from time series of decreasing ferrous concentrations under conditions of varying pH or dissolved oxygen.

ESI 3. R script for linear regression analyses

The R scripts used for linear regression analyses are provided at:

github.com/JoeHeffron/BacteriophageFerrousOxidation

ESI 4. Summary of regression models

Regression models were used to test the fit of experimental bacteriophage inactivation data to the extent of iron oxidation, ferrous iron dose, and ferrous oxidation rate, as shown in Table ESI.3. Similarly, the effect of flocculation time and iron dose was related to particle size via regression, as shown in Table ESI.4.

Table ESI.3: Summary of regression model variables and statistics for bacteriophage inactivation as a function of iron oxidation (controlled by time or sodium thiosulfate addition), ferrous iron dose, and iron oxidation rate constant (controlled by hydroxide concentration or dissolved oxygen concentration). Independent variable transformations were used to test the hypothetical relation between log inactivation and ferrous iron oxidation. Models were evaluated by goodness-of-fit and distribution of residuals, in addition to using ANOVA to identify significant variables.

	Percent iron oxidation (timed test)		Percent iron oxidation (sodium thiosulfate test)		Ferrous dose (mg/L Fe)		Ferrous oxidation rate (min^{-1}, varying hydroxide concentration test)		Ferrous oxidation rate (min^{-1}, varying dissolved oxygen test)	
	MS2	P22	MS2	P22	MS2	P22	MS2	P22	MS2	P22
Independent variable transform	None	None	None	None	None	None	Inverse	Inverse	Inverse	Inverse
p-value	6.95 E-10	3.60 E-09	1.15 E-02	5.64 E-07	6.49 E-07	2.13E-04	1.92 E-08	2.38 E-08	5.04 E-04	2.00 E-04
β coefficient/ slope	0.0104	0.0463	0.00847	0.378	0.228	1.14	0.0732	0.0776	0.0177	0.0449
F statistic (degrees of freedom)	128 (1,19)	104.8 (1,19)	10.65 (1,8)	294 (1,7)	965 (1,5)	91.3 (1,5)	146 (1,13)	112 (1,15)	25.44 (1,10)	19.82 (1,22)
R^2_{adj}	0.864	0.839	0.517	0.973	0.994	0.938	0.912	0.874	0.690	0.450

Table ESI.4: Summary of regression model variables and statistics for floc size as a function of flocculation time and ferrous dose. Separate models were developed for low ferrous doses (< 3 mg/L Fe) and high ferrous doses (> 3 mg/L Fe). Beyond 3 mg/L Fe, floc size was not significantly affected by increasing the ferrous dose. The estimated slope of the time variable (β) was also nearly twice as great at higher ferrous doses, indicating that flocs form more slowly at low doses than at high doses.

	Flocculation time (min)				Ferrous dose (mg/L Fe)				
	transform	β	p-value	F statistic (degrees of freedom)	transform	β	p-value	F statistic (degrees of freedom)	R^2_{adj}
< 3 mg/L Fe	Square root	196	< 2E-16	415 (1, 48)	None	310	< 2E-16	167 (1, 48)	0.921
> 3 mg/L Fe	Square root	370	< 2E-16	1656 (1, 30)	None	16.8	0.246	1.40 (1, 30)	0.981

ESI 5. Floc formation at varying ferrous iron doses

As shown in Figure ESI.3, floc formation was far more visibly apparent above 2 mg/L Fe.

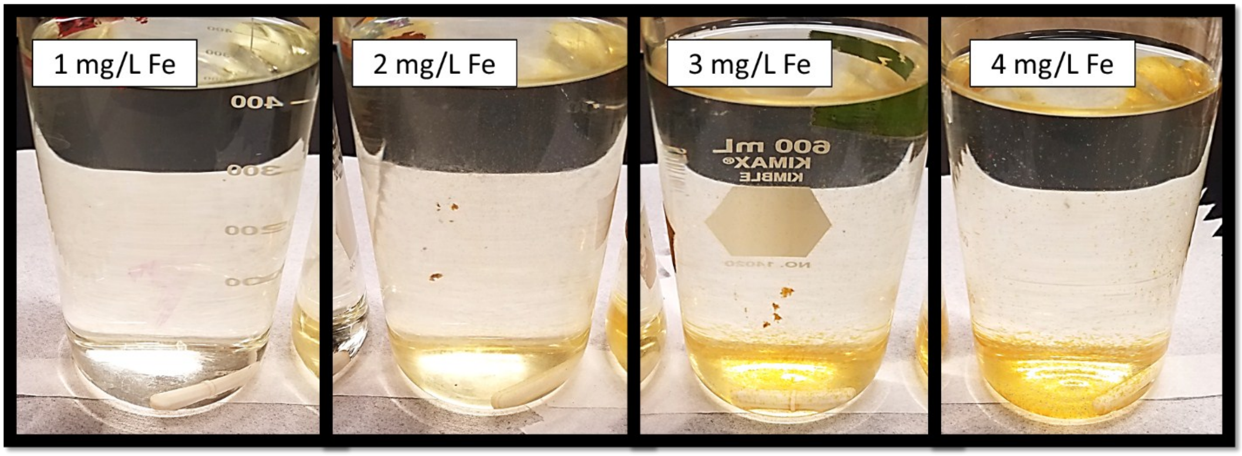

Figure ESI.3: Floc formation at varying ferrous iron doses. Iron was spiked as $FeCl_2 \cdot 4H_2O$ and allowed to form at slow mixing (60 rpm) for 90 minutes. By visual evaluation, abundant, large flocs formed at 3 mg/L Fe and above, while few settleable flocs formed at lower concentrations. These results support the floc formation trends observed via dynamic light scattering.

Floc formation was measured after addition of varying doses of $FeCl_2$ (to 3 mM bicarbonate solution (pH 7). Particle size was measured over the course of 60 min by dynamic light scattering (DLS), as shown in Figure ESI.4. Below approximately 3 mg/L Fe, both the rate of particle formation and the ultimate particle size after 1 h are dependent on ferrous dose. However, at higher doses, particle formation is independent of dose, as confirmed by the regression model shown in Table ESI.4. A manipulatable, 3D surface comparing the predicted model contour to measured data points can be generated using the script "Particle size.R" provided in the GitHub link in ESI 3.

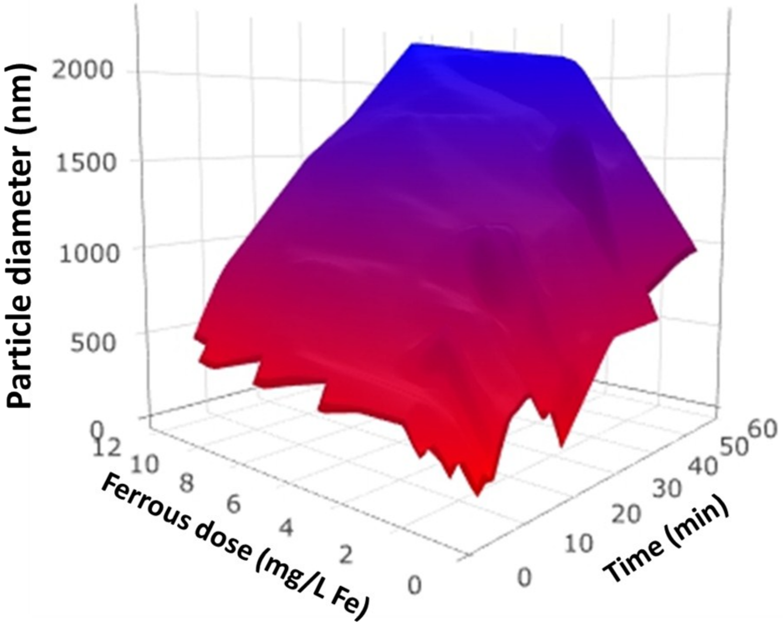

Figure ESI.4: Floc formation over time for varying ferrous doses. The surface shown was interpolated from dynamic light scattering measurements for 84 independent particle diameter measurements.

ESI 6. Impact of ferrous iron oxidation on floc formation

Floc formation was measured after addition of $FeCl_2$ (2.5 mg/L Fe) to 3 mM bicarbonate solution at pH levels ranging from 5.99 to 8.06. Particle size was measured by DLS. Both ferrous oxidation and floc growth was far more rapid at higher pH (Figure ESI.5). Maximum floc size also increased with pH.

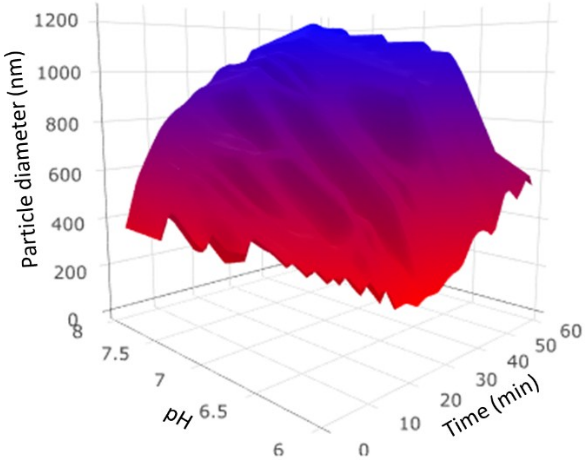

Figure ESI.5: Growth of iron flocs over time as a function of pH. Iron was spiked as $FeCl_2$ (2.5 mg/L Fe) into 3 mM $NaHCO_3$. Surfaces were interpolated from more than 100 data measurements across the ranges shown.

An equilibrium model of ferrous iron speciation over a range of ferric iron concentrations was developed using MINEQL+ software (Environmental Research Software, Hallowell, ME). Experimental conditions were replicated in the model (pH 7, 3 mM bicarbonate solution, $9\text{x}10^{-6}$ M Fe(II), $1.8\text{x}10^{-5}$ M Cl^-). Results are shown in Figure ES1.6.

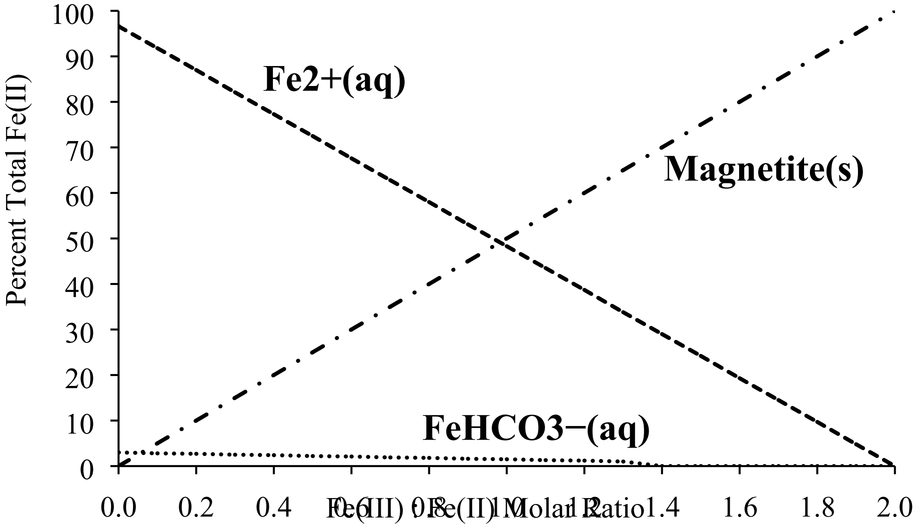

Figure ESI.6: Theoretical ferrous iron speciation as a function of ferric iron concentration. As ferric iron concentration approached the stoichiometric ratio for magnetite ($Fe^{2+}Fe^{3+}_2O_4$), magnetite replaced ferrous ions as the dominant species.