

SUPPLEMENTAL DATA

Determination of Fe(II) oxidation half-life under OECD media conditions

Measurements of Fe(II) oxidation half-life in OECD media buffered with 5 mM MES at pH 6.3, were performed. The half-life was determined with a 5 mg/L Fe(II) sample, as $\text{FeSO}_4 \times 7 \text{ H}_2\text{O}$, following ferrous concentration over time using the ferrozine methodology. The role of an excess of Fe(III) relative to Fe(II) was also considered with a mixture of 9 mg/L Fe(III) and 1 mg/L Fe(II). Oxidation half-life, defined as the time necessary to decrease the Fe(II) concentration to half its initial value due to oxidation was determined according to Brezonik and Arnold¹. Two different exposure scenarios were analyzed, an unstirred scenario and a second mixed scenario (100 rpm), closer to the OECD 72 h growth inhibition test conditions. A summary of the calculated parameters is reported in Table 1.

Table 1. Summary of the kinetic results for the oxidation of Fe(II) species under OECD conditions in the presence of MES 5 mM pH 6.3 at 24 °C. In the first column the initial conditions of the experiment are defined (only Fe(II) or Fe(II) : Fe(III) at 1:9 molar ratio); mixing conditions are presented in the second column; the third column report pH; and half-life ($t_{1/2}$) in the fourth column.

Experiment	Agitation	pH	Half Life, h
Fe(II)	Unstirred	6.3	81
Fe(II)	100 rpm	6.3	58
Fe(II) / Fe(III)	Unstirred	6.3	10
Fe(II) / Fe(III)	100 rpm	6.3	4

Raw data for the measurements of Fe(II) concentration through time, under unstirred or mixed conditions are reported in Table 2 and Figure 1. For the assays in the presence of Fe(III) results are reported in Table 3 and Figure 2.

¹ Brezonik P and Arnold W. (2011). Water Chemistry: An Introduction to the Chemistry of Natural and Engineered Aquatic Systems. Oxford press Inc. pp: 146 – 149.

Table 2. Data sets of Fe(II) concentrations under unstirred and mixed (100 rpm) conditions in OECD media with MES (5 mM) adjusted to pH 6.3. Oxidation time under laboratory atmospheric conditions are reported in the first column; Molar Fe(II) concentration in unstirred conditions in the second column; and Molar Fe(II) concentration in mixed media in the third column.

Time, min	Fe(II)-unstirred, M	Fe(II)-mixed, M
2	7.15E-05	7.24E-05
60	-	7.11E-05
100	-	7.04E-05
180	6.95E-05	-
200	-	6.94E-05
1140	6.06E-05	5.80E-05
1355	5.84E-05	5.49E-05
1620	5.66E-05	5.22E-05
2580	4.96E-05	-

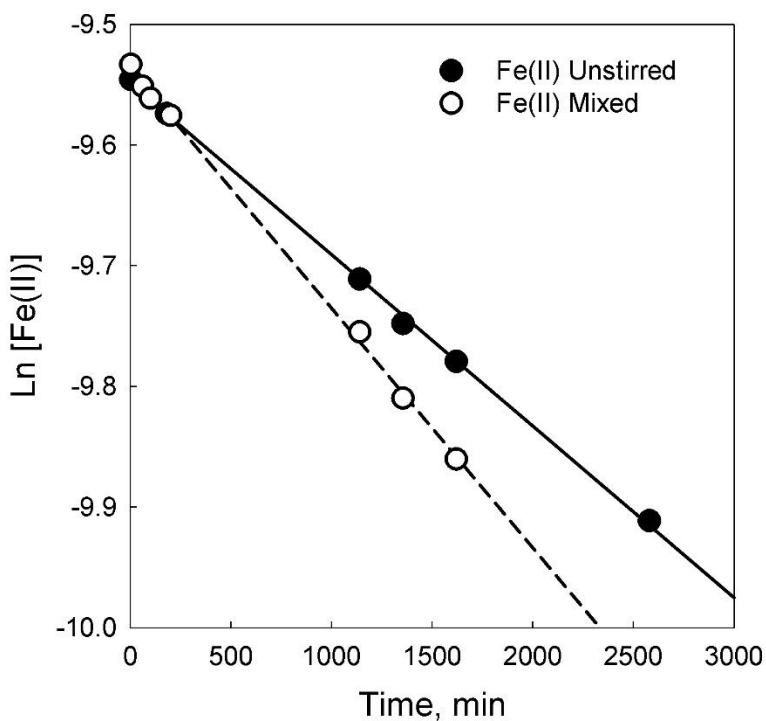


Figure 1. First order plots for the Fe(II) oxidation data in unstirred and mixed conditions are presented. Lines represent the linear regression through the data

Table 3. Fe(II) concentrations under unstirred and mixed (100 rpm) conditions in OECD media with MES (5 mM) adjusted to pH 6.3 and in the presence of a 9:1 Fe(III) to Fe(II) molar ratio. Oxidation time under laboratory atmospheric conditions are reported in the first column; Molar Fe(II) concentration in unstirred conditions in the second column; and Molar Fe(II) concentration in mixed conditions in the third column. Initial concentrations of 1 mg/L Fe(II) and 9 mg/L Fe(III) were used.

Time, min	Fe(II)-unstirred, M	Fe(II)-mixed, M
15	1.54E-05	1.55E-05
71	1.35E-05	1.29E-05
180	1.18E-05	8.77E-06
310	1.03E-05	-
440	9.02E-06	5.07E-06

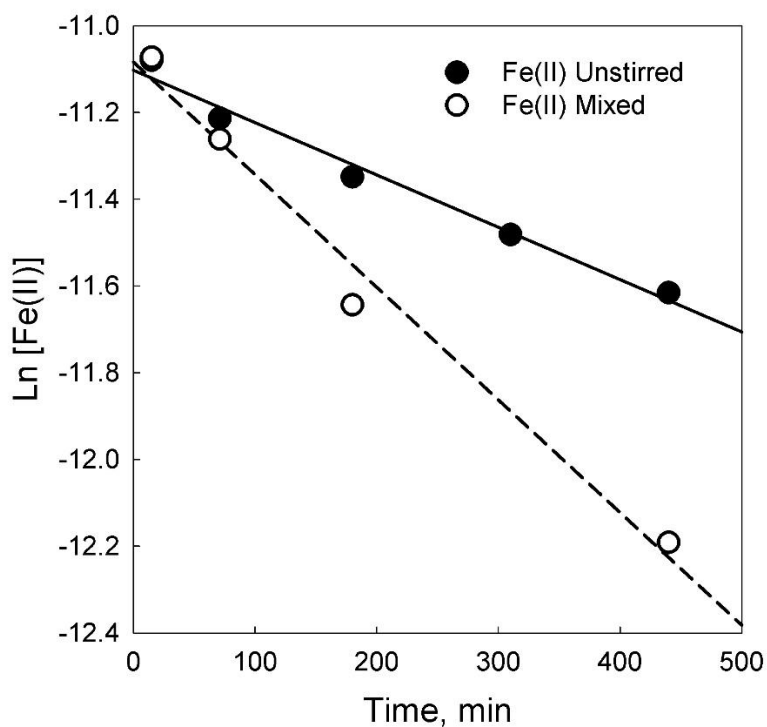


Figure 2. First order plots of Fe(II) oxidation under unstirred and mixed conditions in the presence of a 9:1 Fe(III) to Fe(II) molar ratio, are presented. Lines represent the linear regression through the data.

Phosphorus requirements in the 72 h standard OECD algae test (“No Fe(III), except for Fe(III)-EDTA nutrient was added to the media”)

The phosphorus requirement of *P. subcapitata* in a 72 h OECD test was determined by exposing algae to a range of phosphorous concentrations. This experiment allowed estimation of minimum phosphorus requirements by the algae in a 72 h test. The results of the test are reported in Table 4. Phosphorus concentrations ranged from 0.4 to 11.8 μM , with the highest concentration equivalent to the concentration in standard OECD media.

Table 4. Phosphorus content effect on the growth of *P. subcapitata* in a 72 h OECD ecotoxicity test. First column reports the range of phosphorus concentrations in the test; second and third columns the 72h-EC50 measured as growth rate (r) or yield (y), with the 95% confidence interval; the fourth column media aging time; and the fifth column if phosphorus was added before or after the media was aged.

P content, μM	72h-ErC50, μM	72h-EyC50, μM	Aging	Nutrients
11.8 – 0.4	< 0.4	0.4 (0.2 – 0.7)	3h	added before

Algal yield was inhibited by 50% (i.e., EC50) at 0.4 μM phosphorus and even less if growth rate was considered (Table 4). A plot of yield inhibition against phosphorus content is presented in Figure 3. Raw data can be found in Table 1 of the annex.

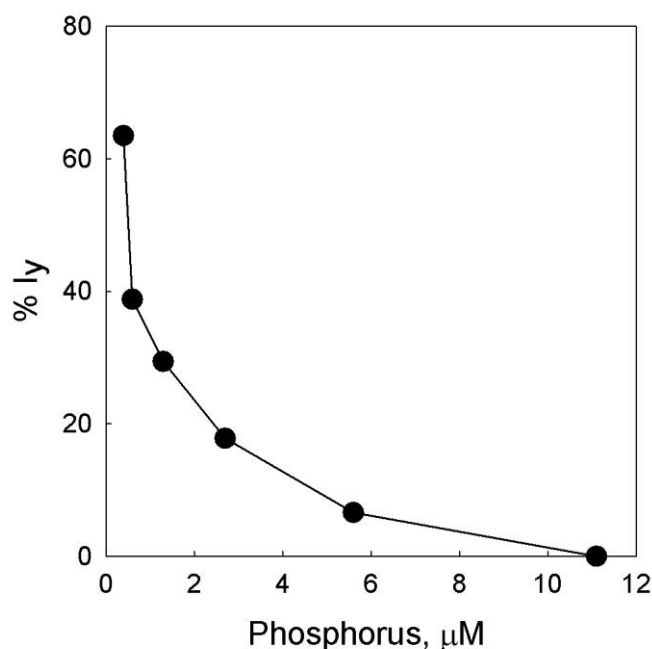


Figure 3. Phosphorus requirements in a *P. subcapitata* 72 h OECD growth inhibition test. Mean percentage inhibition in yield (%I_y) at the end of the test is presented in filled circles.

Effect of DOC and Hardness on Fe(III) toxicity to *P. subcapitata*

1) Comparison of the effect of 4 mg/L DOC in Fe(III) toxicity to *P. subcapitata* in standard OECD media at pH 6.3 or media supplemented with threefold molar excess of phosphorus

The test endpoints reported in Table 5, compared the toxicity results obtained in a standard Fe(III) assay, with the results obtained in the presence of 4 mg/L of DOC or in the presence of 4 mg/L DOC with a threefold phosphorus content at pH 6.3. All the assays were performed under non-filtered conditions (in the presence of Fe(III) oxyhydroxides). The standard toxicity results are reported in the main manuscript (Table 1) and are incorporated here only for comparison purposes.

Table 5. Effect of DOC and threefold phosphorus on Fe (III) toxicity to *P. subcapitata*. First column reports the range of Fe(III) concentrations in the test; second and third columns the 72h-EC50 measured as growth rate (r) or yield (y), with the 95% confidence interval; the fourth column the Fe(III) fraction involved in toxicity; the fifth column the media aging time; in the sixth column a media description (3X-P = threefold phosphorus; DOC = 4 mg/L of dissolved organic carbon); seventh column, pH. All EC50 values are based on total Fe (III) concentrations. In bold, data reported in the publication and added for comparison purposes.

Fe (III) Concentrations	72h-ErC50 (mg/L)	72h-EyC50 (mg/L)	Fe (III) Fraction	Fe (III) Aging	Nutrients	pH
1 – 14.9 mg/L	7.9 (6.9 – 8.9)	3.3 (3.0 – 3.6)	Non filtered	3h	Standard	6.3
1 – 14.5 mg/L	14.2 (13.8 – 14.7)	7.8 (5.4 – 11.4)	Non filtered	3h	DOC	6.3
1 – 14.5 mg/L	> 14.5	> 14.5	Non filtered	3h	DOC + 3X-P	6.3

Detailed characterization of the toxic effect and phosphorus content under both 4 mg/L DOC conditions (normal or threefold molar excess of phosphorus) are presented in Figure 4.

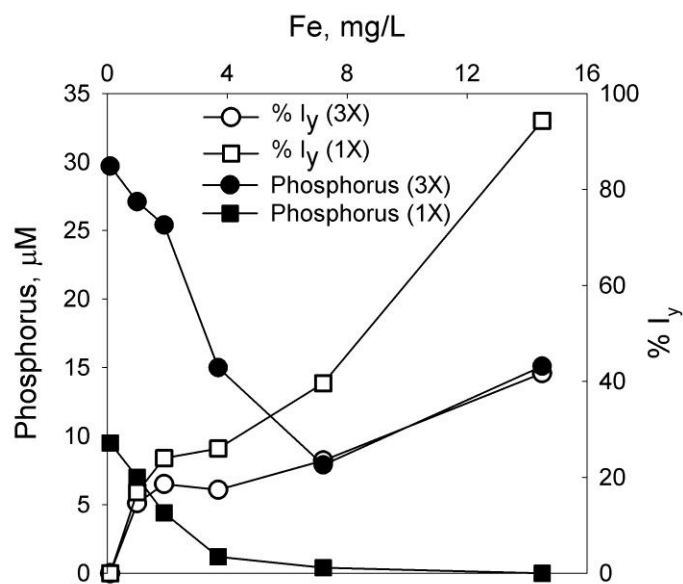


Figure 4. Effect of the addition of DOC and threefold of phosphorus in the toxicity of Fe(III) to *P. subcapitata*. Dissolved phosphorus concentration in the Fe(III) test solutions are shown in filled symbols, and toxicity data presented as yield inhibition in percentage (%I_y), are shown in hollow symbols.

2) Comparison of the effect of 252 mg/L hardness (as CaCO₃) on Fe(III) toxicity to *P. subcapitata* in standard OECD media at pH 6.3 or media supplemented with threefold of phosphorus

The effect of hardness in normal OECD media or a media with a threefold phosphorus content was performed. The test endpoints reported in Table 6 compared the toxicity results obtained in a standard Fe(III) assay, with a hardness of 25 mg/L, against the results obtained with a hardness of 252 mg/L (as CaCO₃). The results of a 72 h Fe(III) toxicity test at a hardness of 252 mg/L and a threefold phosphorus at pH 6.3 was also compared against a similar test that was performed with 25 mg/L hardness. All the tests were performed under non-filtered conditions (in the presence of Fe(III) oxyhydroxides).

Table 6. Effect of hardness and threefold phosphorus in Fe (III) toxicity to *P. subcapitata*. First column reports the range of Fe(III) concentrations in the test; second and third columns the 72h-EC₅₀ measured as growth rate (r) or yield (y), with the 95% confidence interval; the fourth column reports the Fe(III) fraction involved in toxicity; fifth column the Fe(III) media aging time; in the sixth column a media description (3X-P = threefold phosphorus; H 252 = hardness of 252 mg/L as CaCO₃); the seventh column, pH. All EC₅₀ values are based on total Fe (III) concentrations. In bold, data reported in the publication, added for comparison purposes.

Fe (III) Concentrations	72h-ErC ₅₀ (mg/L)	72h-EyC ₅₀ (mg/L)	Fe (III) Fraction	Fe (III) Aging	Nutrients	pH
1 – 14.9 mg/L	7.9 (6.9 – 8.9)	3.3 (3.0 – 3.6)	Non filtered	3h	Standard	6.3
1 – 13.1 mg/L	7.6 (6.6 – 8.8)	2.7 (2.2 – 3.2)	Non Filtered	3h	H 252	6.3
1 – 13.5 mg/L	> 13.5	> 6.9 < 13.5	Non filtered	3h	3X-P	6.3
1 – 14.9 mg/L	> 14.9	10.6 (9.6 – 11.7)	Non Filtered	3h	H 252 + 3X-P	6.3

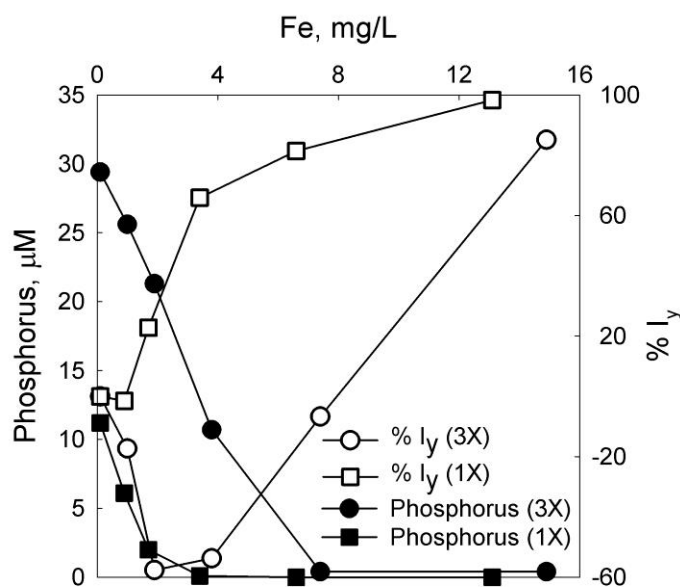


Figure 5. Effect of hardness with and without threefold of phosphorus on the toxicity of Fe(III) to *P. subcapitata*. Dissolved phosphorus concentration in the Fe(III) test solutions are shown in filled symbols, and toxicity data presented as yield inhibition in percentage (%I_y), are shown in hollow symbols.

Annex

Tests Raw Data

Phosphorus requirements in the 72 h standard OECD algae test

Table 1. Results of phosphorus requirements by the algae under OECD conditions, presented as average growth rate and yield.

P (%)	Mean cell density measurement (Cells x 10 ⁴ / mL)				Section by section and entire mean growth rate (days)					Yield	
	0 h	24 h	48 h	72 h	μ (0-1)	μ (1-2)	μ (2-3)	μ (0-3)	%I _r	%I _y	
100	1.0	5.0	22.8	90.0	1.60	1.53	1.36	1.50	0.0	0.0	
50	1.0	5.0	22.7	84.2	1.61	1.51	1.31	1.48	1.3	6.6	
25	1.0	4.7	21.3	74.2	1.53	1.52	1.25	1.44	3.8	17.8	
12.5	1.0	4.5	21.3	63.8	1.50	1.56	1.10	1.40	6.6	29.4	
6.25	1.0	4.3	20.2	55.5	1.46	1.53	1.02	1.36	9.5	38.8	
3.13	1.0	3.7	19.8	33.5	1.29	1.70	0.52	1.22	18.6	63.5	

Effect of DOC and Hardness on Fe(III) toxicity to *P. subcapitata*

1) Comparison of the effect of 4 mg/L DOC on Fe(III) toxicity to *P. subcapitata* in standard OECD media at pH 6.3 or media supplemented with threefold of phosphorus

a) Raw data of ecotoxicity test, micro nutrient and phosphorus concentration in OECD media conditions supplemented with 4 mg/L DOC at pH 6.3.

Table 2-A. Results of Fe (III) toxicity at pH 6.3 in solutions supplemented with 4 mg/L of DOC and aged for 3 hours before test initiation.

Fe (mg/L)	Mean cell density measurement (Cells x 10 ⁴ / mL)				Section by section and entire mean growth rate (days)					Yield	
	0 h	24 h	48 h	72 h	μ (0-1)	μ (1-2)	μ (2-3)	μ (0-3)	%I _r	%I _y	
0.1	1.0	5.5	34.3	141.2	1.70	1.83	1.42	1.67	0.0	0.0	
1.0	1.0	5.7	30.5	117.5	1.73	1.67	1.36	1.60	4.2	16.9	
1.9	1.0	5.7	28.3	107.5	1.73	1.61	1.33	1.56	6.2	24.0	
3.7	1.0	5.3	27.5	104.7	1.67	1.63	1.34	1.56	6.5	26.0	
7.2	1.0	3.3	25.3	85.7	1.19	2.05	1.22	1.54	7.7	39.6	
14.5	1.0	1.7	4.8	9.0	0.50	1.05	0.64	0.76	54.2	94.3	

Table 2-B. Dissolved iron and phosphate concentrations. Method detection limit for Fe = 10 µg/L; P = 0.2 µM.

Iron concentrations (µg/L)			Dissolved Phosphate (µM)		pH	
Total	0 h	72 h	0 h	72 h	0 h	72 h
100	42	33	9.5	0.5	6.35	6.33
1000	227	173	7.0	0.4	6.34	6.33
1900	371	327	4.4	0.8	6.34	6.30
3700	380	286	1.2	0.4	6.30	6.29
7200	324	261	0.4	0.1	6.29	6.30
14500	87	38	0.0	-0.1	6.27	6.29

Table 2-C. Dissolved micro nutrient concentrations (µg/L) at the beginning and end of the test.

Time 0 h (just before algae was added and the test initiated)							
Total Fe, mg/L	Co	Mn	Zn	Mo	Cu	B	Al
0.1	0.40	124	9	3.8	6.2	40	21
1.0	0.42	117	8	3.7	5.9	42	17
1.9	0.45	115	8	3.5	5.7	40	17
3.7	0.50	116	7	3.1	6.1	40	15
7.2	0.59	117	8	1.9	6.2	40	11
14.5	0.87	123	7	0.9	6.4	39	10
Time 72 h (at the end of the test)							
Total Fe, mg/L	Co	Mn	Zn	Mo	Cu	B	Al
0.0	0.40	123	7	3.7	6.1	61	13
1.0	0.40	114	8	3.5	6.8	58	13
1.9	0.44	115	7	3.7	6.0	58	13
3.7	0.48	115	7	3.0	6.0	58	13
6.8	0.58	118	8	2.1	6.5	58	16
14.0	0.84	123	8	1.1	6.5	55	13

b) Raw data of ecotoxicity test, micro nutrient and phosphorus concentration in OECD media conditions supplemented with 4 mg/L DOC and threefold of phosphorus (after 3 hours of aging) at pH 6.3.

Table 3-A. Results of Fe (III) toxicity at pH 6.3 in solutions supplemented with 4 mg/L of DOC and threefold of phosphorus after 3 hours of aging.

Fe (mg/L)	Mean cell density measurement (Cells x 10 ⁴ / mL)				Section by section and entire mean growth rate (days)					Yield
	0 h	24 h	48 h	72 h	μ (0-1)	μ (1-2)	μ (2-3)	μ (0-3)	%I _r	%I _y
0.1	1.0	4.8	32.0	133.8	1.57	1.89	1.43	1.66	0.0	0.0
1.0	1.0	4.7	28.3	114.5	1.54	1.79	1.41	1.60	3.4	14.6
1.9	1.0	4.5	29.2	109.2	1.50	1.87	1.32	1.60	3.8	18.6
3.7	1.0	4.8	29.0	110.7	1.57	1.79	1.34	1.59	4.0	17.4
7.2	1.0	5.0	28.2	102.7	1.61	1.73	1.30	1.56	5.8	23.5
14.5	1.0	4.3	24.2	78.5	1.46	1.70	1.19	1.48	10.8	41.7

Table 3-B. Dissolved iron and phosphate concentrations. Method detection limit for Fe = 10 μg/L; P = 0.2 μM.

Iron concentrations (μg/L)			Dissolved Phosphate (μM)		pH	
Total	0 h	72 h	0 h	72 h	0 h	72 h
100	68	78	29.7	24.3	6.29	6.33
1000	335	555	27.1	21.2	6.27	6.31
1900	907	1054	25.4	17.7	6.26	6.29
3700	536	835	15.0	9.2	6.22	6.28
7200	1430	2468	7.9	4.5	6.26	6.30
14500	7392	443	15.1	0.9	6.25	6.28

Table 3-C. Dissolved micro nutrients concentrations (µg/L) at the beginning and end of the test.

Time 0 h (just before algae was added and the test initiated)							
Total Fe, mg/L	Co	Mn	Zn	Mo	Cu	B	Al
0.1	0.60	167	16	3.7	18.1	40	21
1.0	0.57	163	17	3.4	16.8	38	17
1.9	0.60	217	15	3.3	17.7	37	18
3.7	0.65	158	14	3.2	18.5	39	12
7.2	0.82	168	16	2.8	19.3	37	15
14.5	1.25	196	20	2.1	22.3	38	19
Time 72 h (at the end of the test)							
Total Fe, mg/L	Co	Mn	Zn	Mo	Cu	B	Al
0.0	0.57	182	19	3.3	20.9	48	12
0.9	0.60	178	19	3.3	21.7	48	15
1.9	0.65	180	19	3.3	23.2	49	17
3.5	0.72	180	20	3.3	22.8	52	16
6.6	0.82	169	23	2.8	20.6	59	14
14.3	1.10	179	19	1.1	19.8	63	12

2) Comparison of the effect of 252 mg/L hardness (as CaCO₃) in Fe(III) toxicity to *P. subcapitata* in standard OECD media at pH 6.3 or media supplemented with threefold of phosphorus

a) Raw data of ecotoxicity test, micro nutrient and phosphorus concentration in OECD media conditions with a hardness of 252 mg/L as CaCO₃ at pH 6.3.

Table 4-A. Results of Fe (III) toxicity at pH 6.3 in solutions with a hardness of 252 mg/L.

Fe (mg/L)	Mean cell density measurement (Cells x 10 ⁴ / mL)				Section by section and entire mean growth rate (days)					Yield	
	0 h	24 h	48 h	72 h	µ (0-1)	µ (1-2)	µ (2-3)	µ (0-3)	%I _r	%I _y	
0.1	1.0	4.0	20.5	69.0	1.36	1.65	1.22	1.44	0.0	0.0	
0.9	1.0	4.2	20.2	70.0	1.42	1.58	1.25	1.43	0.2	-1.5	
1.7	1.0	4.3	19.5	53.5	1.46	1.51	1.00	1.34	6.5	22.8	
3.4	1.0	3.2	13.8	24.2	1.14	1.48	0.55	1.10	23.3	65.9	
6.6	1.0	2.0	7.0	13.7	0.67	1.27	0.67	0.91	36.6	81.4	
13.1	1.0	1.3	2.5	2.2	0.27	0.63	-0.17	0.28	80.3	98.3	

Table 4-B. Dissolved iron and phosphate concentrations. Method detection limit for Fe = 10 µg/L; P = 0.2 µM.

Iron concentrations (µg/L)			Dissolved Phosphate (µM)		pH	
Total	0 h	72 h	0 h	72 h	0 h	72 h
100	6	0	11.2	4.7	6.28	6.33
900	37	9	6.1	1.6	6.23	6.32
1700	52	4	2.0	0.0	6.20	6.29
3400	22	5	0.1	0.1	6.19	6.27
6600	41	6	0.0	0.0	6.20	6.29
13100	12	13	0.0	0.0	6.18	6.25

Table 4-C. Dissolved micro nutrients concentrations (µg/L) at the beginning and end of the test.

Time 0 h (just before algae was added and the test initiated)							
Total Fe, mg/L	Co	Mn	Zn	Mo	Cu	B	Al
0.1	0.38	102	11	2.9	8.5	52	3
0.9	0.41	101	10	2.6	8.8	52	3
1.7	0.43	100	11	2.5	10.1	52	3
3.4	0.50	102	21	1.5	15.3	52	4
6.6	0.61	104	11	0.4	11.9	53	4
13.1	0.84	110	11	0.1	12.0	58	4
Time 72 h (at the end of the test)							
Total Fe, mg/L	Co	Mn	Zn	Mo	Cu	B	Al
0.0	0.40	101	14	2.6	11.9	69	3
0.9	0.43	102	11	2.5	10.6	70	3
1.7	0.46	104	12	2.3	11.8	71	3
3.3	0.52	105	11	1.2	11.1	70	3
6.3	0.64	109	10	0.5	9.9	67	3
13.3	0.88	117	11	0.2	11.0	72	3

b) Raw data of ecotoxicity test, micro nutrient and phosphorus concentrations in OECD media conditions with a hardness of 252 mg/L, as CaCO₃, and threefold of phosphorus (after 3 h of aging) at pH 6.3.

Table 5-A. Results of Fe (III) toxicity at pH 6.3 in solutions with a hardness of 252 mg/L and threefold of phosphorus added after 3 h of aging.

Fe (mg/L)	Mean cell density measurement (Cells x 10 ⁴ / mL)				Section by section and entire mean growth rate (days)					Yield	
	0 h	24 h	48 h	72 h	μ (0-1)	μ (1-2)	μ (2-3)	μ (0-3)	%I _r	%I _y	
0.1	1.0	4.7	19.8	73.8	1.54	1.45	1.31	1.44	0.0	0.0	
1.0	1.0	4.7	18.5	66.3	1.54	1.38	1.28	1.40	2.8	10.3	
1.9	1.0	4.3	17.8	65.8	1.46	1.41	1.31	1.40	2.6	11.0	
3.8	1.0	4.8	18.0	64.5	1.57	1.32	1.28	1.38	3.7	12.8	
7.4	1.0	3.8	16.2	56.3	1.34	1.44	1.25	1.35	5.8	24.0	
14.9	1.0	1.8	4.7	13.7	0.60	0.89	1.12	0.87	39.1	82.6	

Table 5-B. Dissolved iron and phosphate concentrations. Method detection limit for Fe = 10 μg/L; P = 0.2 μM. Values between quotes are regarded as outliers due to problems in the filtering stage.

Iron concentrations (μg/L)			Dissolved Phosphate (μM)		pH	
Total	0 h	72 h	0 h	72 h	0 h	72 h
100	19	0	34.2	27.7	6.31	6.32
1000	14	0	29.2	24.1	6.29	6.29
1900	15	5	24.4	18.6	6.29	6.28
3800	14	"95"	16.2	8.2	6.26	6.26
7400	11	26	6.1	0.5	6.26	6.25
14900	9	0	0.1	0.0	6.24	6.22

Table 5-C. Dissolved micro nutrients concentrations (µg/L) at the beginning and end of the test.

Time 0 h (just before algae was added and the test initiated)							
Total Fe, mg/L	Co	Mn	Zn	Mo	Cu	B	Al
0.1	-	-	-	-	-	-	-
1.0	0.45	108	9	2.9	13.1	35	2
1.9	0.48	109	9	2.7	13.4	35	2
3.8	0.52	108	8	2.2	12.4	37	2
7.4	0.62	106	7	1.4	13.9	38	2
14.9	0.84	109	6	0.5	10.8	43	2
Time 72 h (at the end of the test)							
Total Fe, mg/L	Co	Mn	Zn	Mo	Cu	B	Al
0.0	0.39	101	11	2.7	12.4	49	1
0.9	0.41	98	8	2.6	13.2	45	1
1.8	0.43	97	7	2.5	11.5	51	1
3.4	0.46	94	5	2.1	10.1	45	1
6.7	0.56	96	5	1.5	10.0	48	1
13.8	0.77	103	5	0.4	9.1	53	1