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EFFECT OF FE (III) ON *PSEUDOKIRCHNERIELLA SUBCAPITATA* AT CIRCUMNEUTRAL pH IN STANDARD LABORATORY TESTS IS EXPLAINED BY NUTRIENT SEQUESTRATION

JOSE J. ARBILDUA,† GERMAN VILLAVICENCIO,† PAOLA URRESTARAZU,† MARGARET OPAZO,† KEVIN V. BRIX,‡ WILLIAM J. ADAMS,§ and PATRICIO H. RODRIGUEZ*†

†CIMM, Santiago, Chile

‡EcoTox, Miami, Florida, USA

§Rio Tinto, South Jordan, Utah, USA

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Abstract: The complex chemistry of iron (Fe) at circumneutral pH in oxygenated waters and the poor correlation between ecotoxicity results in laboratory and natural waters have led to regulatory approaches for iron based on field studies (US Environmental Protection Agency Water Quality Criteria and European Union Water Framework Directive proposal for Fe). The results of the present study account for the observed differences between laboratory and field observations for Fe toxicity to algae (*Pseudokirchneriella subcapitata*). Results from standard 72-h assays with Fe at pH 6.3 and pH 8 resulted in similar toxicity values measured as algal biomass, with 50% effect concentrations (EC50) of 3.28 mg/L and 4.95 mg/L total Fe(III), respectively. At the end of the 72-h exposure, however, dissolved Fe concentrations were lower than 30 μg/L for all test concentrations, making a direct toxic effect of dissolved iron on algae unlikely. Analysis of nutrient concentrations in the artificial test media detected phosphorus depletion in a dose-dependent manner that correlated well with algal toxicity. Subsequent experiments adding excess phosphorus after Fe precipitation eliminated the toxicity. These results strongly suggest that observed Fe(III) toxicity on algae in laboratory conditions is a secondary effect of phosphorous depletion. *Environ Toxicol Chem* 2017;36:952–958. © 2016 SETAC

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INTRODUCTION

Iron is one of the most abundant elements in the earth's crust and is also an essential element, required by all living organism to maintain their homeostasis and growth. Iron is essential in plants for the biosynthesis of chlorophyll and as a donor/acceptor of electrons in the respiratory chain. In vertebrates and some invertebrates, iron is an integral component of oxygen transport, DNA synthesis, and immune responses to pathogens [1–3]. Iron depletion in some alkaline natural waters has been described to be a limiting factor for the growth of algae and other plants; similarly, iron is known to be a limiting factor for oceanic primary producers [3,4].

Iron speciation in oxygen-rich media at circumneutral pH is dominated by Fe (III), with a half-life of the ferrous species, Fe (II), on the order of minutes to hours [5–7]. The fast hydrolysis and polymerization of the Fe (III) species results in the formation of metal colloids that aggregate in a time-dependent process. Different Fe species have been characterized during this process, including low molecular mass products, a red cationic hydrolytic polymer, and mineralized species formed through aging (i.e., goethite) [8]. Factors such pH, pO_2, dissolved organic carbon (DOC), redox potential, temperature, and suspended particles can affect Fe bioavailability to aquatic organisms [1,9–12].

From a regulatory perspective, iron is considered a nonpriority pollutant by the US Environmental Protection Agency (USEPA), with a recommended water quality criterion of 1 mg/L for continuous chronic exposure, a value that has been in use since 1976 [3]. The criterion is based primarily on field studies, because of the lack of correlation in toxicity between laboratory and field studies. In contrast, under the Water Framework Directive, the United Kingdom Technical Advisory Group has proposed [2] a new iron quality standard in freshwaters that, if approved, will be in effect for river basin management from 2015–2021. This proposal considers total iron concentration, with a threshold of 0.73 mg/L and, as with the USEPA iron criterion, it is also based primarily on field evidence. Field data analyzed for fish, macrophyte, and diatom communities indicate no decline in ecological quality with increasing total iron exposure at pH above 7.0; however, benthic macroinvertebrate were sensitive to increasing iron exposure [2]. Regulation based exclusively on field data has some important drawbacks. First, it is difficult to extrapolate the field or mesocosm studies to waters with different physicochemical characteristics. In the proposed water quality standards (WQS) for Fe, empirical relationships among DOC concentrations, water hardness, and Fe toxicity are recognized; however, insufficient data are available to apply these relationships to waters with a pH below 7.0 [2]. Second, it is difficult to demonstrate unequivocally the cause of a toxic effect in field studies, as this type of study is not performed under controlled physicochemical conditions and concentrations of other stressors (e.g., aluminum) may correlate with Fe concentrations [13].

Information on iron toxicity to aquatic organisms is, in general, scarce [1–3]; Fe (II) is considered more toxic than Fe (III), and is responsible for direct effects on animal survival. Higher concentrations of Fe (II) can be found in anoxic groundwaters and under acidic conditions such as drainage of peatlands and leaching of iron-rich ores. In contrast, Fe (III)

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* Address correspondence to patricio.rodriguez@cimm.cl

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species dominate oxygenated waters at circumneutral pH and are associated with indirect toxicity to organisms via metal hydroxide precipitation, limiting their access to nutrients, impairing respiration, or interfering with movement [1,3,12].

To reduce uncertainty regarding iron toxicity under different physiochemical conditions, it would be useful to complement field studies with data from laboratory studies using artificial waters, to account for physicochemical conditions poorly represented in field studies. Therefore, it is important to understand the mechanisms underlying the differences in Fe toxicity between laboratory and natural waters. The present study reports the effect of Fe(III) on the algae *Pseudokirchneriella subcapitata* in synthetic laboratory waters (controlled conditions) with the aim of identifying the mechanism(s) of Fe (III) toxicity to algae.

MATERIALS AND METHODS

Synthetic assay media

Pro-analysis grade chemicals were obtained from Merck. The synthetic assay media was prepared following guideline 201 of the Organisation for Economic Co-operation and Development (OECD) [14]. To establish a pH in the media of 6.3 or 8, 2 synthetic buffers were used: 2-(N-morpholino) ethane-sulfonic acid (MES; 5 mM) or N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid (HEPES; 5 mM), respectively, purchased from Sigma-Aldrich. Ferric sulfate n-hydrate was obtained from J.T. Baker. Standard dissolved organic matter (Suwannee River natural organic matter, isolated by reverse osmosis) was obtained from the International Humic Substances Society. Dilution water was generated using a Millipore Elix-100/Super-Q system and is capable of producing water with less than 1 μg/L of total metals and less than 0.1 mg/L of total organic carbon.

P. subcapitata *growth inhibition tests*

Pseudokirchneriella subcapitata inoculum was taken from an aseptic 4-d-old preculture in exponential growth. Volumes of the inoculums were between 0.5 mL and 1 mL for each 100 mL of test media. The test substance was added as ferric sulfate n-hydrate ($Fe_2[SO_4]_3 \cdot nH_2O$) from a 1 g/L Fe (III) solution freshly prepared. Test solutions spiked with Fe were aged for 3 h prior to test initiation to allow Fe to reach approximate chemical equilibrium. Growth inhibition tests were initiated by addition of algae to the individual test solutions. An inoculum containing 10 000 cells/mL of *P. subcapitata* was added to each Erlenmeyer glass flask (250 mL), containing 100 mL of test or control culture media, and placed on an orbital shaker. Each individual test included 5 Fe(III) test concentrations plus a control (untreated) with 3 replicates per treatment. Tests were conducted without media renewal for 72 h under continuous fluorescent illumination, and the light intensity was in the range of 86 ± 8.6 μE/m^2/s, as recommended by the protocol [14]. The temperature was maintained at 24 ± 2 °C, and a maximum gas exchange and suspension of the cells was ensured by the action of the orbital shaker oscillating at 100 cycles/min.

To discriminate between chemical and physical effects of Fe(III) in the tests, some of the assays were performed using treatments that were filtered through 0.45-μm polyvinylidene difluoride (PVDF) membranes (diameter, 4.7 cm; Millipore). Filtration was performed after 3 h of aging and prior to the addition of the algae, to avoid the presence of Fe oxyhydroxides in the incubation media.

Growth rates based on daily cell density of the treatments over the 72-h exposure period were determined by microscopic counting using a Neubauer improved chamber from HBG.

Data analysis

Average growth rates in the control and test treatments were calculated as the logarithmic increase in biomass during the test period (expressed per day) in accordance with OECD protocols [14]. Yield was calculated as the algal biomass at the end of the test minus the starting biomass for each flask (control and treatments). The median effect concentrations based on growth rate and biomass yield (72-h ErC50 and 72-h EbC50, respectively) were estimated by nonlinear regression analysis, using the USEPA's Toxicity Relationship Analysis Program (TRAP), Ver 1.00.

Analytical measurements

Metal concentrations were measured using a PerkinElmer inductively coupled plasma–mass spectrometer (ICP–MS) model ELAN 9000. To ensure the quality of metal measurements, 2 quality control standards, QC1 and QC2, were used. The QC1 standard was prepared based on the QCS-26 solution, and the QC2 was based on the QCS-27 solution (cocktails of 26 and 27 metals, respectively, both at a concentration of 100 mg/L; from High-Purity Standards for ICP-MS). The tolerances of our measurements were within 20% of the certified value. If the measured metal concentration for the reference material or standard did not fall within 20% of the certified value, a new calibration of the instrument was performed, and the samples were analyzed again. For total metal measurements, 10-mL aliquots were collected at 0 h (before the addition of algae) and at the end of the 72-h exposure. Then the samples without algae were directly acidified with 1% nitric acid (Merck Suprapur) and stored at 4 °C. For the samples with algae, acid digestion according to USEPA method 3015A [15] was performed before analysis. Dissolved metal measurements were performed by collecting 10-mL aliquots at 0 h and at the end of the 72-h exposure. Then the samples were filtered through 0.45-μm PVFD syringe-filters and acidified with 20 μL of sulfuric acid in 15-mL polypropylene tubes. Samples were stored at 4 °C until they were analyzed.

Spectrophotometric iron measurements were carried out using the formation of a colorimetric complex between Fe(II) and ferrozine (3-[2-pyridyl]-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt; Sigma-Aldrich P9762-5G) measured at 562 nm. Briefly, for total iron determination, 7.8-mL samples were reduced with 0.1 mL of 50 mM ascorbic acid, to convert Fe(III) to Fe(II) under acidic conditions (pH between 1 and 3) for 5 min; then 0.1 mL of 1 M acetate buffer at pH 5.5 was added to the sample, and complexation with ferrozine was performed by the addition of 2 mL of a 10 mM solution dissolved in 0.1 M ammonium acetate. The reaction was left to complete for 5 min before the sample absorbance was measured using an HP 8452A diode array spectrophotometer [16,17]. Ferrous iron was measured with the same methodology as total iron but without the reduction step with ascorbic acid.

Phosphorus measurements were performed according to USEPA method 365.3 [18]. The colorimetric assay forms an antimony–phospho–molybdate complex of an intense blue color after reduction by ascorbic acid. Absorbance was determined at 716 nm using an HP 8452A diode array spectrophotometer. A quantification limit for phosphorus of 0.2 μM was calculated.

The DOC concentration of stock solutions was measured using USEPA method 415.1 [19] with a Tekmar Dohrmann, Apollo 9000 carbon analyzer. The DOC content was not measured in the test vessels because of the presence of buffers (carbon based).

RESULTS AND DISCUSSION

Fe (III) toxicity to P. subcapitata

Iron (III) toxicity has usually been attributed to an indirect effect because of the presence of Fe oxyhydroxides species, which have an adverse effect on the health of aquatic organisms by physical or mechanical action [12,20,21]. A simple way to evaluate whether the toxicity of Fe(III) to algae is attributable to a direct toxic effect or to the action of Fe oxyhydroxides is to compare the toxicity results obtained from the exposure of the algae with increasing concentrations of the metal under filtered and unfiltered conditions (i.e., in the presence and absence of Fe oxyhydroxides). Therefore, 4 independent tests were performed: 2 tests at pH 6.3 and 2 tests at pH 8.0. One of the assays had all test solutions filtered through 0.45-μm PVDF membranes before the algae were added and the test began, and the other assay was performed without filtration of the media (with Fe oxyhydroxides present).

Similar toxicity results, reported as EC50 values, were found for *P. subcapitata* at both pH values under filtered and unfiltered conditions (Table 1). All the calculated EC50 values based on biomass were between 2.82 mg/L and 4.95 mg/L (total iron). The EC50 values based on growth rate were higher and exhibited a broader toxicity range, between 5.95 mg/L and 16.70 mg/L (total iron). The similar effects observed in filtered and unfiltered tests suggest that Fe(III) toxicity to *P. subcapitata* is not the result of a physical effect associated with the hydrolysis products of Fe(III), but is rather of a chemical nature.

Dissolved Fe concentrations measured in the different assays at the different total Fe doses measured at 0 h and 72 h (Table 2) were relatively constant—at approximately 20 μg/L in the pH 6.3 tests and approximately 40 μg/L in the pH 8.0 tests—and showed no correlation with the observed effects on the algae. In principle, 2 alternative hypotheses for Fe(III) toxicity to the algae may be formulated. First, the toxicity is produced by a very toxic soluble Fe species that is a subpopulation of the dissolved Fe, and as such correlates with the toxicity of total Fe (III). However, this hypothesis seems unlikely because of the low and relatively constant dissolved iron concentrations found in the media. Second, Fe (III) toxicity may be the result of nutrient (Fe or another nutrient) depletion via Fe coprecipitation. If insoluble Fe(III) species are able to coprecipitate nutrients, higher total Fe concentrations will result in greater removal of nutrients and nutrient deficiency. In this hypothesis, toxicity will correlate to total Fe(III) concentrations, and the effect will be observed in both the filtered and unfiltered media, as was observed in the tests.

A potential role of Fe(II) in the observed toxicity to algae in this pH range was discounted for several reasons. First, Fe(II) in the solutions were analyzed using the ferrozine method, and less than 0.07% ferrous impurity was found. Second, we measured the oxidation rate of Fe(II) under current media conditions and observed a half-life of 2.4 d at pH 6.3. This value decreased to 4 h in the presence of Fe(III), at a ratio of 1:9 (Fe(II):Fe(III)), probably because of the adsorption of Fe(II) on the surface of Fe oxyhydroxides [22], which may lead to a faster oxidation process or Fe(II) sequestration from solution (Supplemental Data, Table S1 and Figures S1 and S2). The oxidation rate at pH 8.0 was not measured; but, on the basis of other observations [5], the half-life is expected to be on the order of minutes.

Fe(III)-induced nutrient depletion

To test the nutrient depletion hypothesis, a 72-h test was performed at pH 6.3 in which the Fe(III) test solutions were prepared and aged for 3 h and then filtered through a 0.45-μm PVDF membrane; then all nutrients were added to the test solutions after the filtration step to prevent potential nutrient coprecipitation with Fe. No toxicity was observed in this experiment (Table 3), suggesting that Fe(III) toxicity is the

Table 1. Summary of filtered and unfiltered Fe(III) ecotoxicity tests on *Pseudokirchneriella subcapitata*, with Fe solutions aged for 3 h before test initiation[a]

pH value	Fe (III) mg/L	Filtered Media Mean cell density (Cells × 10^4/mL)				Unfiltered Media Mean cell density (Cells × 10^4/mL)			
		0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
6.3	0.1	1.0	5.8	23.7	107.5	1.0	4.5	18.2	69.5
	1.2	1.0	5.0	28.2	108.7	1.0	4.5	21.2	73.8
	2.1	1.0	5.2	27.0	104.3	1.0	4.5	19.8	60.3
	4.1	1.0	4.8	8.8	11.2	1.0	2.0	9.3	22.2
	7.9	1.0	4.2	6.3	7.7	1.0	1.3	3.0	8.5
	14.9	1.0	4.3	4.0	6.2	1.0	1.2	2.0	3.2
	72h-ErC50 (95% CI)	5.95 (3.99 – 8.89)				7.86 (6.91 – 8.94)			
	72h-EbC50 (95% CI)	2.82 (2.24 – 3.54)				3.28 (3.03 – 3.56)			
8.0	0.1	1.0	4.0	25.8	137.7	1.0	5.8	22.0	95.3
	1.0	1.0	4.2	24.0	115.7	1.0	4.8	21.8	79.0
	1.9	1.0	4.8	23.8	113.8	1.0	3.7	20.5	78.0
	3.7	1.0	4.7	25.0	79.7	1.0	3.8	15.8	56.2
	7.1	1.0	4.2	17.8	14.8	1.0	2.5	9.8	37.2
	14.3	1.0	4.8	13.0	10.8	1.0	1.8	5.3	11.5
	72h-ErC50 (95% CI)	11.75 (9.68 – 14.27)				16.70 (15.30 – 18.23)			
	72h-EbC50 (95% CI)	4.18 (3.75 – 4.66)				4.95 (4.28 – 5.73)			

[a]Two pH conditions were evaluated, pH 6.3 and pH 8. The 72-h median effective concentration (EC50) values (mg/L) measured as growth rate (ErC50) and yield (EbC50) are given with the 95% confidence interval (CI) in parentheses. Hardness was 25 mg/L, as $CaCO_3$. All EC values are based on total Fe(III) concentrations.

Table 2. Total and dissolved Fe concentrations in filtered and unfiltered ecotoxicity tests on *Pseudokirchneriella subcapitata*, with Fe solutions aged for 3 h before test initiation[a]

pH	Total Fe (III) mg/L	Filtered media: Dissolved Fe (0 h; μg/L)	Filtered media: Dissolved Fe (72 h; μg/L)	Unfiltered media: Dissolved Fe (0 h; μg/L)	Unfiltered media: Dissolved Fe (72 h; μg/L)
6.3	0.1	12	<10	17	15
	1.2	16	<10	29	13
	2.1	12	<10	26	<10
	4.1	11	<10	15	<10
	7.9	14	<10	13	<10
	14.9	<10	<10	13	<10
8.0	0.1	33	21	59	24
	1.0	36	23	89	28
	1.9	39	31	80	25
	3.7	38	33	46	27
	7.1	35	38	45	26
	14.3	36	41	40	26

[a]Two pH conditions are evaluated, pH 6.3 and pH 8. Dissolved Fe concentrations were measured at time 0 (before algae was added) and after 72 h. Hardness was 25 mg/L, as $CaCO_3$.

result of the adsorption/coprecipitation of one or more essential nutrients.

A comparison of nutrient concentrations at the beginning (before addition of the inoculum, at time 0 h) and at the end of a Fe(III) toxicity test (72 h) in the presence of Fe oxyhydroxides reveals a decrease over time of the concentration of phosphorus and molybdenum (Table 4). Also, the observed decrease in dissolved Fe concentrations below detection limits after 72 h suggests that Fe(III)–ethylenediamine tetraacetic acid (EDTA) may be coprecipitating with the hydrolysis products of Fe(III), leading to an iron-deficient media.

Additional 72-h tests were carried out in which the test media solutions were supplemented with either of the 2 nutrients, Fe (III)–EDTA or phosphorus, after the filtration step (Table 5). The EC50 values in the toxicity test supplemented with Fe(III)–EDTA were comparable to those in the nonsupplemented test. In contrast, toxicity was completely eliminated in the toxicity test supplemented with phosphorus, suggesting that phosphorus depletion is the main mechanism of action for the observed effect of Fe(III) on algae biomass and growth.

To confirm the role of phosphorus in the observed effect of Fe(III) on algae, a 72-h test in the presence of Fe oxyhydroxides and an excess of phosphorus was conducted. The experiment included all of the nutrients in the test solutions, which were aged for 3 h in the presence of Fe(III), followed by the addition of a 2-fold molar excess of phosphorus to the aged media already containing 11.8 μM phosphorus. Growth rate was not inhibited by 50% over the range of concentrations tested, and a significant increase in the EbC50 was observed (Table 6). Because effects were observed only at the highest concentration, the TRAP software program was unable to estimate a 72-h EbC50, but the value was between 6.9 mg/L (16% reduction in biomass) and 13.5 mg/L (83% reduction in biomass) total Fe, based on visual inspection of the data. The addition of excess phosphorus had at least 2 clear consequences. First, it increased the dissolved Fe(III) by approximately an order of magnitude (Table 6), suggesting either that the phosphorus interacts with aggregates competing with Fe(III) or that the increased

Table 3. Summary of Fe(III) ecotoxicity tests on *Pseudokirchneriella subcapitata* in test solutions supplemented with media nutrients, pH 6.3[a]

Fe(III) (mg/L)	Dissolved Fe (μg/L) 0 h	Dissolved Fe (μg/L) 72 h	Mean cell density (cells × 10^4/mL) 0 h	24 h	48 h	72 h
0.1	32	16	1.0	5.0	21.2	83.3
1.0	32	15	1.0	5.3	25.7	118.5
2.0	26	15	1.0	5.0	21.5	104.2
3.9	30	16	1.0	5.0	21.5	98.0
7.2	29	15	1.0	4.3	25.0	122.8
13.4	28	16	1.0	4.3	27.7	126.7

72-h ErC50 >13.4 mg/L
72-h EbC50 >13.4 mg/L

[a]Fe solutions were aged for 3 h before the test solutions were filtered through 0.45-μm poly (1,1,2,2-tetrafluoroethylene) membranes and all nutrients were supplemented to the filtrate. The 72-h test was performed at pH 6.3 in the presence of 2-(N-morpholino) ethane-sulfonic acid (5 mM) and a hardness of 25 mg/L, as $CaCO_3$. The 72-h median effective concentration (EC50) values (mg/L) measured growth rate (ErC50) and yield (EbC50), were reported. All EC values are based on total Fe(III) concentrations.

Table 4. A 72-h test was performed at pH 6.3 in the presence of MES (5 mM) and a hardness of 25 mg/L, as $CaCO_3$[a]

Total Fe	Fe	P	B	Co	Cu	Mn	Mo	Zn
	Dissolved metal concentration (μg/L)							
0 h; mg/L								
0.1	17	301	42	0.41	6.3	126	3.8	6
1.2	29	178	38	0.41	6.6	113	3.7	6
2.1	26	62	38	0.43	6.7	114	3.7	7
4.1	15	<6	38	0.50	6.5	116	2.3	7
7.9	13	<6	37	0.60	6.6	117	1.1	7
14.9	13	<6	37	0.84	6.3	119	0.8	5
72 h; mg/L								
0.0	15	176	61	0.41	5.6	127	4.0	6
0.9	13	11	55	0.43	6.5	115	3.8	6
1.8	<10	<6	58	0.45	6.1	116	3.4	6
3.5	<10	6	53	0.51	6.8	118	2.1	6
6.5	<10	<6	59	0.63	6.4	120	1.0	6
13.7	<10	<6	59	0.88	6.6	125	0.8	6

[a]Fe solutions were aged for 3 h before the test initiation (not filtered). Total and dissolved Fe concentrations were measured at time 0 h (before algae was added) and after 72 h. Nutrient levels in the tests solutions at time 0 h and after the end of the 72-h test were measured.
MES = 2-(N-morpholino) ethane-sulfonic acid.

Table 5. Summary of Fe(III) ecotoxicity tests on *Pseudokirchneriella subcapitata,* using test solutions supplemented with Fe(III)–EDTA or phosphorus[a]

Fe(III) (mg/L)	Dissolved Fe (μg/L) 0 h	Dissolved Fe (μg/L) 72 h	Dissolved P (μg/L) 0 h	Dissolved P (μg/L) 72 h	Mean cell density (cells × 10^4/ mL) 0 h	24 h	48 h	72 h
Media supplemented with Fe(III)–EDTA after filtration								
0.0	15	23	350	84	1.0	4.3	21.7	102.3
0.9	29	24	202	16	1.0	4.5	21.0	86.2
1.7	33	25	78	<6	1.0	4.3	18.7	101.8
3.5	35	26	6	<6	1.0	4.5	8.8	10.7
6.6	36	20	<6	<6	1.0	3.3	4.7	4.8
12.8	40	26	<6	<6	1.0	3.2	4.0	3.7
72-h ErC50 (95% CI) 4.34 (3.37–5.59)								
72-h EbC50 (95% CI) 2.30 (2.06–2.57)								
Media supplemented with phosphorus after filtration								
0.0	16	<10	344	192	1.0	4.2	19.3	79.8
0.8	14	<10	347	102	1.0	4.5	20.3	99.3
1.6	14	<10	350	93	1.0	4.7	19.7	104.8
3.2	13	<10	347	47	1.0	4.3	22.0	113.8
6.3	13	<10	357	37	1.0	4.0	28.0	122.5
12.4	<10	<10	350	50	1.0	4.3	25.8	118.7
72-h ErC50 >12.4 mg/L								
2-h EbC50 >12.4 mg/L								

[a]Fe solutions were aged for 3 h before the test solutions were filtered through 0.45-μm poly (1,1,2,2-tetrafluoroethylene) membranes and the missing nutrient supplemented to the filtrate for the 2 tests. The 72-h tests were performed at pH 6.3 in the presence of 2-(N-morpholino)ethane-sulfonic acid (5 mM) and a hardness of 25 mg/L, as CaCO3. The 72-h median effective concentration (EC50) values (mg/L) were measured as growth rate (ErC50) and yield (EbC50), with the 95% confidence intervals (CIs) in parentheses. All EC values are based on total Fe(III) concentrations.
EDTA = ethylenediamine tetraacetic acid.

phosphorus concentration is able to disaggregate the hydrolysis products of Fe(III) into smaller particles. Similar observations have been reported in natural waters, where the Fe(III) content in the colloidal fraction (between 12 kDa and 0.45 μm) increased in the presence of phosphorus [23,24]. These *dissolved* (<0.45 μm) Fe(III) and phosphorus concentrations were stable for days. Second, it ensured that sufficient phosphorus was available for algal growth throughout the 72 h at all but the highest Fe(III) level (Table 6). Therefore, phosphorous depletion in the test solutions correlated with the inhibition of algal growth.

Table 6. Summary of Fe(III) ecotoxicity test on *Pseudokirchneriella subcapitata,* with an excess of phosphorus added to the test solutions after the 3-h aging period without filtering[a]

Fe(III) (mg/L)	Dissolved Fe (μg/L) 0 h	Dissolved Fe (μg/L) 72 h	Dissolved P (μg/L) 0 h	Dissolved P (μg/L) 72 h	Mean cell density (cells × 10^4/ mL) 0 h	24 h	48 h	72 h
0.0	95	95	958	787	1.0	5.7	22.8	85.8
0.9	141	100	862	725	1.0	4.5	22.0	72.7
1.8	224	125	775	549	1.0	5.5	20.0	75.5
3.5	145	113	570	307	1.0	6.3	22.8	68.0
6.9	91	98	276	31	1.0	4.5	19.5	72.3
13.5	373	115	37	9	1.0	1.5	3.0	15.0
72-h ErC50 >13.5								
72-h EbC50 >6.9 <13.5								

[a]The effect of an excess of phosphorus in media containing Fe oxyhydroxides was evaluated. The 72-h test was performed at pH 6.3 in the presence of 2-(N-morpholino) ethane-sulfonic acid (5 mM) and a hardness of 25 mg/L, as $CaCO_3$. The 72-h median effective concentration (EC50) values (mg/L) were measured as growth rate (ErC50) and yield (EbC50). All EC values are based on total Fe(III) concentrations.

The interaction of phosphorus with Fe(III) is a process well documented in natural waters, with Fe oxyhydroxides and organic matter playing a role in the bioavailability of phosphorus. Studies have proposed that this interaction is a functional mechanism for phosphorus mobilization/sequestration in aquatic systems. The sequestration of phosphorus and other nutrients by Fe(III) has been used as a eutrophication control tool in natural waters [24–28].

Influence of DOC and hardness on the toxicity of Fe(III) to P. subcapitata

The indirect Fe(III) effect on algae described in the *Fe(III) induced nutrient depletion* section appears to be an artifact of the test protocol. However, phosphorus sequestration by Fe(III) usually occurs in the environment, when Fe-rich groundwater meets aerated surface waters and the Fe(II) oxidation process allows the formation of Fe hydroxyphosphate precipitates [28].

The test protocol in synthetic laboratory water used in the present study (OECD guideline 201 [14]) lacks the complexity of natural waters, with associated phenomena such as photoreduction via DOC complexation and ultraviolet (UV) light, anoxic environments, phytoplankton reduction and consumption of iron, particulate matter transporting metals and nutrients, and temperature oscillations [11,12]. However, metal toxicity is usually influenced by the physicochemical characteristics of natural waters, especially by the formation of organic complexes with DOC and competition with other cationic species like calcium and magnesium (i.e., water hardness). To evaluate the potential effect of hardness and DOC on the indirect mechanism of Fe(III) toxicity to algae, 2 separate 72-h tests were carried out, 1 at a hardness of 252 mg/L as $CaCO_3$ and 1 in the presence of 4 mg/L of DOC, both after aging for 3 h with no filtration.

Comparison of the results obtained at high (252 mg/L) versus low (25 mg/L; same pH) hardness shows that, in contrast to

Table 7. Summary of Fe(III) ecotoxicity tests on *Pseudokirchneriella subcapitata* conducted at a hardness of 252 mg/L or in the presence of 4 mg/L of dissolved organic carbon (DOC)[a]

Fe(III) (mg/L)	Dissolved Fe (μg/L)		Dissolved P (μg/L)		Mean cell density (cells × 10^4/mL)			
	0 h	72 h	0 h	72 h	0 h	24 h	48 h	72 h
Hardness of 252 mg/L, as $CaCO_3$								
0.1	<10	<10	347	146	1.0	4.0	20.5	69.0
0.9	37	<10	189	50	1.0	4.2	20.2	70.0
1.7	52	<10	62	<6	1.0	4.3	19.5	53.5
3.4	22	<10	<6	<6	1.0	3.2	13.8	24.2
6.6	41	<10	<6	<6	1.0	2.0	7.0	13.7
13.1	12	13	<6	<6	1.0	1.3	2.5	2.2
72-h ErC50 (95% CI) 7.60 (6.59–8.76)								
72-h EbC50 (95% CI) 2.67 (2.23–3.20)								
DOC 4 mg/L								
0.1	42	33	295	17	1.0	5.5	34.3	141.2
1.0	227	173	217	12	1.0	5.7	30.5	117.5
1.9	371	327	137	25	1.0	5.7	28.3	107.5
3.7	380	286	38	13	1.0	5.3	27.5	104.7
7.2	324	261	14	<6	1.0	3.3	25.3	85.7
14.5	87	38	<6	<6	1.0	1.7	4.8	9.0
72-h ErC50 (95% CI) 14.23 (13.78–14.69)								
72-h EbC50 (95% CI) 7.83 (5.38–11.40)								

[a]Fe concentrations were aged for 3 h before the algae was added to tests solutions and the assay initiated. The 72-h tests were performed at pH 6.3 in the presence of 2-(N-morpholino) ethane-sulfonic acid (5 mM). The 72-h median effective concentration (EC50) values (mg/L) measured growth rate (ErC50) and yield (EbC50), with the 95% confidence intervals in parentheses. All EC values are based on total Fe(III) concentrations.

other metals, such as copper [29], hardness does not provide a large protective effect for Fe(III) toxicity to algae. The 72-h EbC50 of 2.67 mg/L (expressed as total Fe(III) concentration) at 252 mg/L (Table 7) was similar to the value obtained at 25 mg/L hardness (3.28 mg/L; Table 1). For growth rate, the 72-h ErC50 of 7.60 mg/L at 252 mg/L hardness was similar to the value at 25 mg/L hardness (7.86 mg/L).

In contrast, 4 mg/L DOC in the media did have a protective effect. The 72-h EbC50 was 7.83 mg/L, compared with 3.28 mg/L for the no-DOC case (Table 7). Dissolved Fe concentrations increased approximately by an order of magnitude in the test solutions compared with tests in the absence of DOC, indicating that Fe is in a form that can pass through the 0.45-μm filter membrane. Initial phosphorus concentrations in the test solutions again correlated with observed effects on algae.

Two additional 72-h tests were performed to determine whether algal growth was able to recover in the presence of excess phosphorus in the unfiltered media, under high hardness or in the presence of DOC. In both cases, significant increases in EC50 values were observed (Supplemental Data, Table S5).

Factors influencing the effect of Fe(III) in artificial laboratory waters

The results of the present study agree with the literature in that the effect of Fe(III) on aquatic organisms is associated with indirect nocive effects of Fe oxyhydroxides (e.g., blocking access to nutrients, impairment of respiration, interference with movement) [1,3,12,20,21]. In the particular case of the Fe(III) effects on algae in laboratory standard assays, the present study shows that the mechanism of action is mediated by phosphorus depletion, through coprecipitation with the hydrolysis products of Fe(III).

It can be concluded that the effect of Fe on *P. subcapitata* in reconstituted laboratory waters depends largely on the level of phosphorus in the assay media as well as the presence of DOC. In contrast, hardness and pH (6.3 or 8) have minor effects on Fe toxicity under the conditions tested. None of these factors are currently explicitly considered in any Fe regulation. In the proposed WFD guideline for Fe, which is based on total Fe concentrations, only pH is indirectly considered, while hardness and DOC are only discussed in terms of ensuring the most sensitive exposure conditions [2,3]. From a regulatory viewpoint, the results of the present study show that iron toxicity in standard laboratory conditions cannot be considered an intrinsic property of the element, and therefore this type of microalgae assay should not be used for the hazard assessment of Fe.

Do any of the processes studied in these experiments have environmental relevance for natural waters? The background concentration of Fe in natural waters ranges from <1 mg/L for aerated surface waters to approximately 90 mg/L for Fe-rich groundwater [28] (i.e., well within the toxic thresholds found in the present study). Phosphorus, on the other hand, is a common agricultural pollutant, and the Fe–P sequestration processes described in the present study also occur in nature and may play a positive role in decreasing eutrophication [23,25,28].

Some observations in aquatic ecosystems suggest the possible effect of Fe–P interactions on toxicity. For example, in circumneutral streams affected by Fe precipitation, a decrease in the number of benthic species, especially grazers, has been observed as a result of the absence of periphyton [2,30]. It would be interesting to study whether the absence of periphyton is causally linked to phosphorus depletion induced by the presence of Fe oxyhydroxides.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3609.

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Data Availability—Data, associated metadata, and calculation tools are available in the online Supplemental Data.

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