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Indirect effects of iron oxide on stream benthic communities: capturing ecological complexity with controlled mesocosm experiments

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Abstract

Ferric iron (Fe(III)) oxyhydroxides commonly precipitate at neutral pH and in highly oxygenated conditions in waterways receiving acid mine drainage, degrading stream benthic communities by smothering habitat, primary producers, and aquatic invertebrates. Stream mesocosms were used to expose naturally colonized benthic communities to a gradient of ferric Fe (0-15 mg/L) for 14 d to estimate the effects of Fe precipitates on primary production, larval and emerging adult aquatic insects, and macroinvertebrate community structure. Community composition was significantly altered at concentrations near or below the USEPA chronic Fe criterion (1.0 mg/L). Iron exposure significantly decreased larval and emerging adult abundances of Baetidae (mayfly) and Chironomidae (Diptera); however, while Simuliidae (Diptera) larvae were not reduced by the Fe treatments, abundance of emerged adults significantly decreased. Iron substantially decreased the colonization biomass of green algae and diatoms, with estimated EC20 values well below the Fe criterion. In contrast, cyanobacteria were stimulated with increasing Fe concentration. By integrating environmentally realistic exposure conditions to native benthic communities that have complex structural and functional responses, the ability to predict the effects of Fe in the field is improved. Traditional toxicity testing methodologies were not developed to evaluate indirect effects of contaminants, and modernized approaches such as community mesocosm experiments better characterize and predict responses in aquatic ecosystems outside the laboratory. Therefore, the development of water quality standards would benefit by including mesocosm testing results.

Introduction

Iron (III) oxyhydroxide precipitates occur in streams receiving acid mine drainage (AMD) under neutral pH and highly oxygenated conditions. Although the dissolved ferrous (Fe^{2+}) form of iron (Fe) is more soluble and is directly toxic to aquatic organisms, ferric iron (Fe^{3+}) is generally considered indirectly toxic due to physical habitat alteration^{1,2} In lotic systems, ferric Fe (hereafter referred to as Fe) can fill interstitial space (i.e., habitat for benthic organisms), impair primary production (e.g., smother periphyton, decrease light penetration), and reduce benthic invertebrate abundance and richness.³⁻⁷ The US Environmental Protection Agency (USEPA) chronic criterion for Fe (1.0 mg/L total recoverable) was primarily developed with field surveys of trout in a small stream in Colorado.⁸ Outside of the US, lower freshwater Fe criterion (0.3 mg/L) have been adopted by Canada,⁹ Australia and New Zealand.¹⁰ Although adverse effects typically do not occur at the USEPA criterion concentration in the laboratory, and Fe is generally considered relatively non-toxic,^{11,12} field surveys have demonstrated effects to benthic communities below the USEPA criterion.¹³

Discrepancies between laboratory-predicted Fe toxicity and field observations are likely due to differences in the effects of Fe at dissimilar spatiotemporal scales. For example, most laboratory approaches are short-duration and do not allow sufficient time for Fe deposition and accumulation into interstitial spaces.¹ Also, many commonly employed test species are pelagic (e.g., *Daphnia magna*, *Pimephales promelas*) and can avoid Fe deposition, or they are adapted to fine sediment environments similar to deposited Fe precipitates (e.g., *Chironomus riparius*). These test species do not adequately represent the diversity of life histories that naturally occur in stream benthic communities.^{14,15} In contrast, field approaches are somewhat limited because they usually do not control confounding and extraneous variables.¹⁶ For instance, Fe generally

co-occurs with other metals at AMD sites, temporal differences in water velocity influence rates of Fe deposition, and the presence of other anthropogenic stressors limits the ability to isolate the effects of Fe.¹⁷⁻¹⁹

Stream mesocosms provide a unique combination of ecological realism and experimental control, addressing many of the shortcomings of laboratory and field evaluations of Fe oxides.² Mesocosm experiments can test the responses of native benthic communities that contain numerous taxa with complex life histories.²⁰⁻²² Exposure to Fe under conditions that include natural benthic habitat and continuous flow better simulate the dynamics of Fe deposition in streams compared to most laboratory approaches. These experiments can test the direct and indirect effects of contaminants on primary production and can investigate potential trophic cascades that may occur between benthic invertebrates (i.e., consumers) and autochthonous primary producers (i.e., food resources),²³ while still controlling most physical and chemical characteristics of the exposure system. Importantly, mesocosms have reconciled field and laboratory results for other metals, often with benthic invertebrates responding at lower concentrations compared to single-species assessments.²⁴⁻²⁶

Evaluations of benthic communities typically examine immature life stages (e.g., larvae) of aquatic insects to characterize taxa sensitivity, yet most aquatic insects metamorphose to a winged-terrestrial adult life stage to reproduce and complete their life cycle. Emerged adults are crucial for population persistence, and they provide prey subsidies to linked riparian consumers.^{27,28} However, the transition from larvae to the adult life stage is biologically stressful because of the high rates of cell differentiation and energy expenditure during tissue reorganization.²⁹ Field and laboratory studies of aquatic insect emergence have shown greater sensitivity of emerging adults to dissolved trace metals compared to larvae.^{30,31} Therefore, the

evaluation of larval life stages alone risks underestimating the effects of metals on aquatic insects and the export of subsidies to linked-terrestrial ecosystems.³⁰⁻³³ Many commonly used laboratory test species do not metamorphose to an adult life stage (e.g., *Daphnia* spp., *Ceriodaphnia dubia*); thus, these tests lack an equivalent endpoint to evaluate the effects of Fe oxyhydroxides on metamorphosis and emergence. Furthermore, most studies conducted with aquatic insects fail to enumerate emerged adults as they emigrate from testing systems during exposure.

To test the effects of Fe oxide on stream communities, we exposed naturally-colonized benthic communities to a gradient of ferric Fe in stream mesocosms. The Fe exposure simulated natural conditions that maintained constant Fe concentrations in the water column while interstitial space of the benthic habitat filled with Fe precipitates. Specific goals of this study were: 1) to quantify effects of Fe deposition on primary production by measuring algal colonization; 2) to compare lethal and sublethal effects of Fe among aquatic insect taxa and between developmental life stages (i.e., larvae versus emerged adult); and 3) to determine concentrations of Fe that significantly alter benthic community structure and to identify taxa that most contributed to community differences among treatments.

Methods

A mesocosm experiment was conducted at the Stream Research Laboratory (SRL) located at Colorado State University Foothills Campus, Fort Collins, Colorado, USA. Naturally colonized stream benthic communities were collected from the Arkansas River (AR; Leadville, Colorado, USA), a high-elevation (~ 3000 m ASL) 4th-order mountain stream (39.1283 N, 106.3111 W). Communities were collected from the AR using a colonization technique described by Clements et al.³⁴ Plastic trays (10 x 10 x 6 cm) filled with pebble and small cobble

were deployed in the AR for 30 d and colonized with a diverse benthic assemblage (collected August 26, 2015). The trays were colonized by a range of life stages of aquatic insects that included developmentally immature taxa, as well as taxa nearing the completion of their life cycle as emerged adults. Five trays were randomly placed into each of 18 insulated coolers, transported to the SRL, transferred to 20-L stream mesocosms, and assigned Fe treatments.

Water quality in the SRL is representative of cold water Rocky Mountain streams with cool temperatures (10-15 °C); low hardness (30-35 mg/L CaCO₃), alkalinity (25-30 mg/L CaCO₃), conductivity (60-90 µS/cm) and concentrations of dissolved organic carbon (2.5-3.0 mg/L); and circumneutral pH (7.0-7.8). Current in the 18 mesocosms was driven by submersible water pumps (EcoPlus®) at a rate of 2233 L/h (i.e., 1.9 hydraulic circulations/min). On day 0, four 25-cm² unglazed ceramic tiles were placed in each mesocosm directly downstream of the colonization trays to estimate algal colonization. All tiles were pre-soaked in bleach for 24 h and rinsed multiple times with reverse osmosis water before deployment. Each mesocosm was covered with insect mesh netting to capture emerged adults (**Fig. S1**). Emerged adults were aspirated from the netting every evening between 17:00 and 19:00 and preserved in 80% ethanol. To prevent emigration by drifting invertebrates, standpipes in the stream mesocosms were covered with fine mesh.

Each mesocosm received dilution water at 1.0 L/min while peristaltic pumps delivered 10 mL/min Fe stock solution. To convert all Fe(II) to Fe(III), ferric chloride stock solutions in 20-L carboys for each stream were vigorously aerated while NaOH was added to attain a pH greater than 6.5. After 60 min of stirring, pH was reexamined before dosing was initiated. Target Fe treatment concentrations were 0.9, 1.8, 3.7, 7.5, and 15.0 mg/L. To maintain a homogenous suspension of Fe oxyhydroxide precipitates in the carboys, stock solutions were vigorously

aerated throughout the exposure. Total (unfiltered) and dissolved (0.45- μ m filtered) water samples were taken from each mesocosm on 4 dates, acidified to a pH < 2.0 using analytical grade nitric acid to dissolve all Fe, and analyzed by flame atomic absorption spectrophotometry. In addition, ferrous Fe concentration was measured by colorimetric analysis using a ferrous Fe reagent (1,10 -phenanthroline indicator; Hach method 8146. Loveland, CO, USA). Temperature, pH, conductivity, and turbidity were measured every 3 d (YSI Pro1030). Alkalinity and hardness were measured by titration (USEPA methods 200.7; 310.7). To estimate deposition of Fe, 50 mL beakers containing 20 mL of glass beads (6-mm diameter) were placed in each stream mesocosm. The Fe was allowed to settle in the interstitial spaces of the beads for 96 h, then the contents were transferred to polypropylene jars and rinsed with ultrapure water. Samples were passed through a 5-mm sieve (to remove beads) and filtered through a vacuum funnel containing a pre-weighed 10- μ m glass fiber filter (GC50, Sterlitech). The filter and Fe precipitates were then dried and weighed.

After 10 d, we estimated algal colonization (on ceramic tiles) with a BenthosTorch (bbe Moldaenke GmbH, Germany), an *in situ* pulse-amplitude modulated fluorometer that measures benthic algae biomass as chlorophyll *a* and estimates the relative contribution of diatoms, green algae, and cyanobacteria pigment complexes to total algal biomass.³⁵ On day 11, we estimated community metabolism (defined as the difference between photosynthesis and whole community respiration) using diurnal changes in dissolved O₂ concentrations in the water column (YSI ProODO. Yellow Springs, OH, USA). After 14 d, colonization trays were rinsed through a 350- μ m sieve, and organisms and detrital material were preserved in 80% ethanol. All larvae were enumerated to the lowest taxonomic resolution possible, typically to genus, except for chironomids that were identified to subfamily or tribe. Emerged adult insects were enumerated

and identified to family or subfamily. For biomass estimates, larval and adult taxa were dried at 60 °C for 72 h and weighed to the nearest 0.00001 g.

Statistical Analyses

All univariate statistical analyses were conducted using R statistical computing.³⁶ We used a linear model (“LM” function; package ‘car’) to test significant concentration-response relationships between Fe and algae community composition and metabolism.³⁷ Non-linear concentration response curves were fit for the algal data using Sigma Plot Ver. 11. A linear model was used to test concentration-response relationships between life-stage abundances and biomass of the three dominant taxa that had sufficient densities of larvae *and* adults ($n > 15$) (Baetidae, Chironomidae, and Simuliidae) and total macroinvertebrate abundance. To determine if the concentration-response relationships of larvae and adults were statistically different, an ANCOVA interaction model was used (“LM” function), with Fe as the continuous predictor and life stage as the categorical predictor of either abundance or biomass. Statistical significance of the interaction term indicates that the slopes developed for larvae and adults differed (i.e., the effect of Fe was dependent on life stage). Lastly, for all significant linear models we estimated EC20 values (effect concentration that reduced the endpoint by 20%, exclusive of control data) with two-parameter log-logistic concentration-response curves. The only exception was for cyanobacteria colonization that required a two-parameter log-normal concentration-response model because biomass increased with Fe concentration (package ‘drc’).³⁸ EC50 values were also calculated to compare to EC20 estimates (**Fig. S2**); however, we chose to report EC20s based on the USEPA risk-management decision to derive Final Chronic Values using EC20

values.³⁹ Concentration-response models were selected based on Akaike Information Criterion (“mselect” function) and visual model fit for all endpoints.

To test for differences in the timing of adult emergence, Fe treatments were categorized (5 treatments, 3 replicates each) to support repeated-measures ANOVA analyses. Daily observations were combined to cumulative emergence, and abundance of emerging adults was summed over the duration of the experiment. We combined daily observations because we wanted to test if the difference in the number of emerging individuals over the entire experiment differed among treatments. A linear mixed model (“lmer” function; package ‘lme4’) was fit to the cumulative emergence data to test if the effects of Fe on cumulative emergence varied during the exposure.⁴⁰ We chose a covariance structure of compound symmetry, which assumes constant correlation between observations on the same sample replicate (i.e., cumulative observations are dependent on the previous observation). *Post hoc* comparisons with “estimated marginal means of linear trends” function from package ‘emmeans’ were used to compare treatment means of emergence abundance to control values with Dunnett’s Test.⁴¹ All data were log transformed to meet the assumptions of parametric statistics and to improve model fit.

Multivariate analyses (PRIMER-e v7; + PERMANOVA; Quest Research Limited, Cambridge, United Kingdom) were used to test for changes in aquatic insect community composition among Fe treatments.⁴² Larval and adult abundance were log (x+1) transformed and a Euclidean distance matrix was calculated. Rare taxa were removed based on the *a priori* decision to define rare species as less than 10 individuals in any treatment. Treatments were categorized, and a one-way permutational multivariate analysis of variance (PERMANOVA) was run with 999 permutations to test for significant alterations in community composition. Pairwise-comparisons were used to test for differences between treatments and controls. Because

of the relatively low number (10) of ‘Unique Permutations’ generated (i.e., the number of unique values of the test statistic obtained under permutation), Monte Carlo adjusted *p*-values were calculated by random sampling from the asymptotic permutation distribution. A Similarity Percentages (SIMPER) analysis was performed on the log-transformed data to determine which taxa accounted for greater than 50% of the dissimilarity among the Fe treatments compared to the controls. Lastly, a nonmetric-multidimensional scaling (NMDS) plot was used to visualize differences among treatments in ordination space.

Results

Measured total Fe concentrations ranged from 0.85 to 14.85 mg/L Fe and were within 10% of the target values (**Table 1**). Among all treatments, filtered Fe and ferrous Fe concentrations were below the detection limit. Water hardness and alkalinity were approximately 31 mg/L CaCO₃ (s.d. = 0.55, n = 12) and 28 mg/L CaCO₃ (s.d. = 1.01, n = 12), respectively. Temperature and pH showed little variation and were consistent among treatments. Specific conductance (77.6 to 195 µS/cm) and turbidity (1.6 to 9.7 NTU) increased with metal concentration. Rates of deposition in all treated mesocosms were significantly correlated with total Fe measured in the water column ($R^2 = 0.86$; **Fig. S3**).

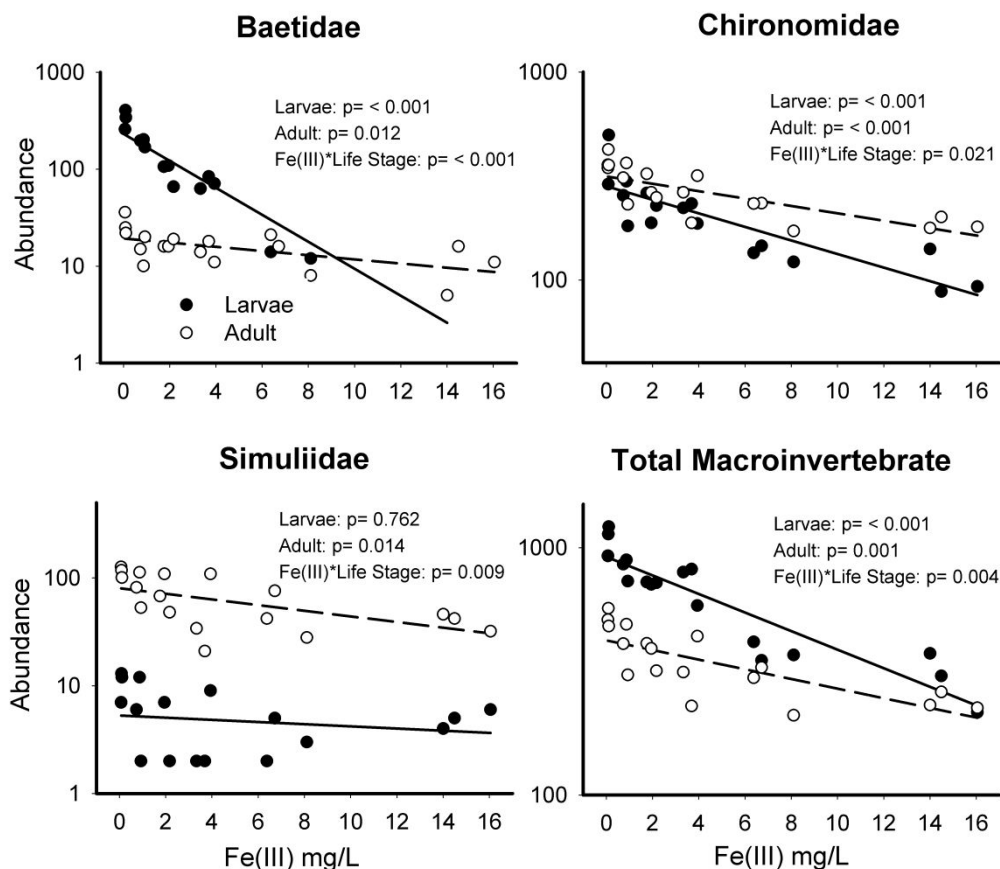
Table 1: Water quality characteristics in stream mesocosms. Values are reported as mean (\pm s.d., n = 12). Fe detection limit using flame atomic absorption spectrophotometry was 0.06 mg/L.

Fe(III) Treatment	Target (mg/L)	Dissolved Fe		Temperature (°C)	pH	Conductivity (µS/cm)	Turbidity (NTU)
		Fe (mg/L)	Total Fe (mg/L)				
Control	0	bdl	0.08 (0.02)	14.9 (0.5)	7.43 (0.18)	77.6 (1.9)	1.6 (0.1)
Low	0.94	bdl	0.85 (0.10)	14.7 (0.3)	7.42 (0.14)	86.9 (3.6)	2.3 (0.2)
Mid-Low	1.88	bdl	1.95 (0.21)	14.9 (0.6)	7.42 (0.12)	93.6 (1.6)	3.1 (0.2)
Mid	3.75	bdl	3.65 (0.30)	14.7 (0.3)	7.4 (0.07)	107.9 (2.9)	3.9 (0.1)

Mid-High	7.5	bdl	7.06 (0.91)	14.4 (0.4)	7.37 (0.07)	133.4 (7.8)	5.4 (0.8)
High	15	bdl	14.85 (1.06)	14.3 (0.4)	7.39 (0.04)	195.9 (14.4)	9.7 (0.4)
<hr/>							
230							

231 The benthic community was diverse, with control mesocosms averaging (\pm s.e., $n = 3$) 28
232 (± 1) taxa and 1093 (± 86) individuals per stream (**Table S1**). Number of taxa and abundance of
233 emerging adults in controls averaged 8 (± 0.3) and 520 (± 25), respectively. Three dominant
234 taxonomic groups (Baetidae, Chironomidae and Simuliidae) were present at sufficient larval and
235 adult densities in controls for statistically valid comparisons. Baetidae, Chironomidae, and total
236 larval and emerging adult abundance significantly ($p < 0.10$) decreased as Fe concentration
237 increased (**Fig. 1; Table S2**). The abundance of larval Simuliidae was unaffected by Fe
238 treatments, but emergence was significantly reduced. For all dominant groups and total
239 community abundance, the ANCOVA interaction term was significant, indicating that the
240 response to Fe was dependent on life stage (**Table S3**). The effects of Fe were greater on larval
241 survival than emergence for Baetidae, Chironomidae and total community abundance, but
242 Simuliidae emerged adults were more sensitive to Fe than larval responses. Among significant
243 linear models, EC20 values ranged from 0.01 (Baetidae adult abundance) to 1.01 mg/L Fe
244 (Chironomidae adult abundance), respectively (**Table S4**). Lastly, the cumulative emergence of
245 the 3 dominant groups was significantly altered during the 14 d exposure (**Fig. 2**) with decreased
246 emergence relative to controls (**Table S5**).

247



248

249 **Figure 1.** Effects of Fe on total larval (solid circles) and emerging adult (open circles)
 250 abundances of Baetidae (Ephemeroptera), Chironomidae (Diptera), Simuliidae (Diptera), and the
 251 total macroinvertebrate community after the 14 d exposure. Linear models for larvae (solid line)
 252 and adults (dashed line) were used to determine significant concentration-response relationships
 253 for each life stage. ANCOVA model was used to test the hypothesis that the slopes for larvae and
 254 adults differed, as indicated by the p-value for the interaction term ($\text{Fe(III)} * \text{Life Stage}$).

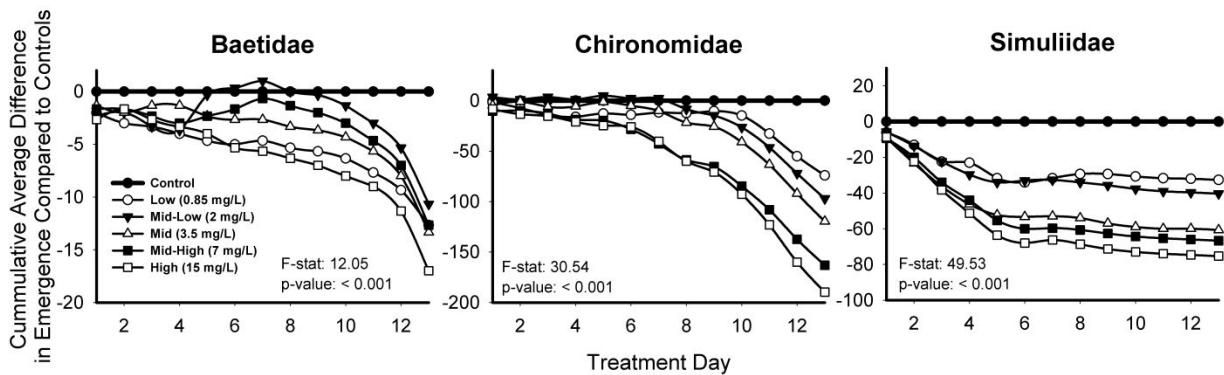


Figure 2. Effects of Fe on the cumulative emergence of Baetidae (Ephemeroptera), Chironomidae (Diptera), and Simuliidae (Diptera) during the 14 d exposure. Single-factor repeated measures ANOVA was used to test for significant differences in cumulative emergence among treatments during the exposure.

Across the Fe treatment gradient, biomass of adult mayflies, chironomids and simuliids was greater than larval biomass after the 14 d exposure. Effects of Fe on biomass of baetids and simuliids (**Fig. 3**) were generally similar to those observed for abundance (**Fig. 1**). However, patterns in biomass of chironomids (larvae and adults) and total macroinvertebrate biomass differed from those observed for abundance, with similar effects of Fe on these group's biomass reduction regardless of life stage (i.e., slopes for larvae and emerged adults were not significantly different) (**Table S3**). EC20 values ranged from 0.001 mg/L Fe for Baetidae adult biomass to 0.80 mg/L Fe for Chironomidae adult biomass (**Table S4**).

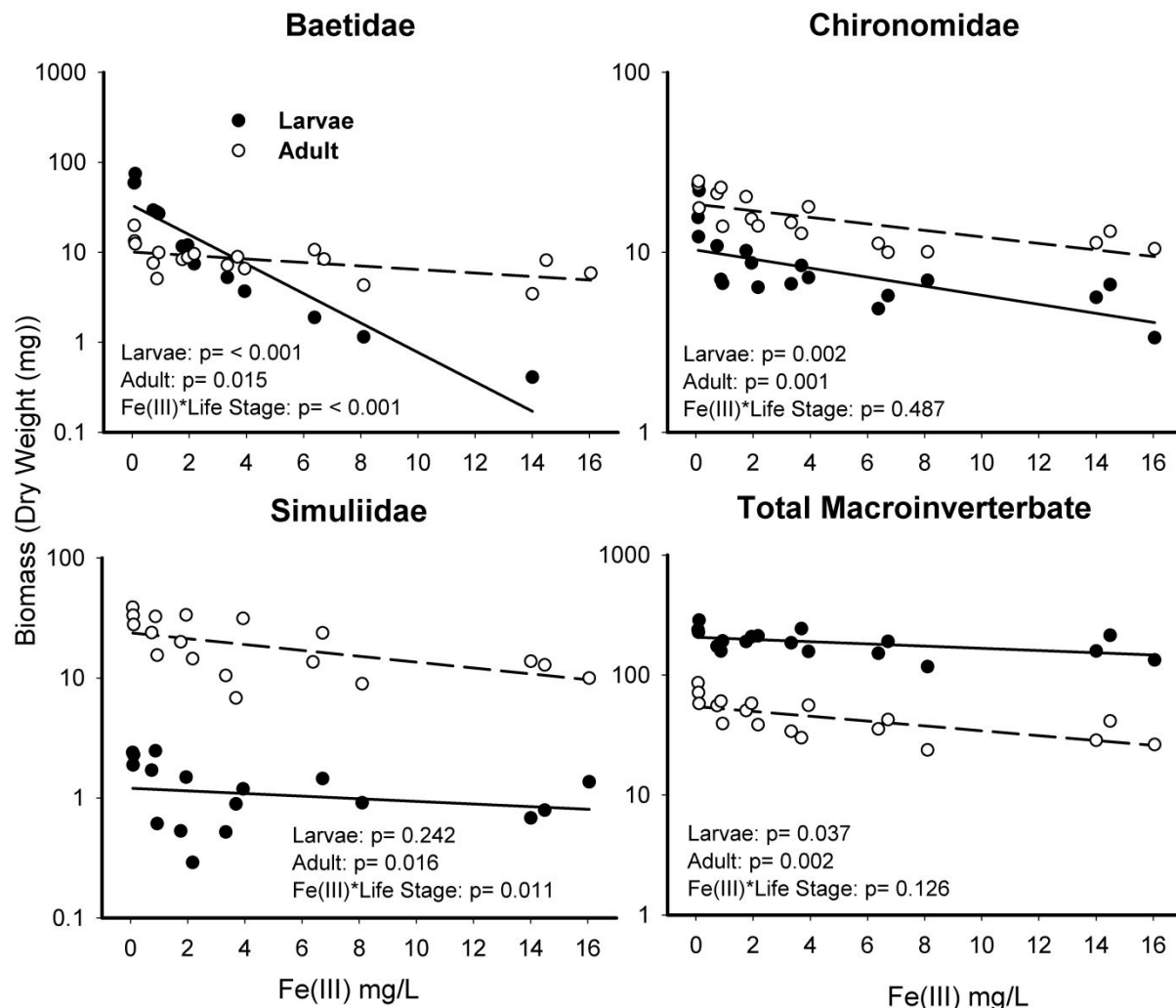


Figure 3. Effects of Fe on total larval (solid circles) and emerging adult (open circles) biomass of Baetidae (Ephemeroptera), Chironomidae (Diptera), Simuliidae (Diptera), and the total macroinvertebrate community in the 14 d community exposure. Linear regression for larvae (solid line) and adults (dashed line) was used to determine significant concentration-response relationships for each life stage. ANCOVA model was used to test the hypothesis that the slopes for larvae and adults differed, as indicated by the p-value for the interaction term (Fe(III)*Life Stage).

Fe significantly altered community composition based on PERMANOVA results (**Table S6**). Visual inspection of the NMDS plot showed clear separation among Fe treatments compared to controls, with increased separation as Fe concentration increased (**Fig. 4**). The three control mesocosms were very close in NMDS space (i.e., similar community structure), whereas separation within Fe treatments was much greater, suggesting that exposure to Fe increased variability in community composition. Pairwise comparisons between each of the treatments and the controls showed substantial alterations in community composition (**Table 2**), including significant alterations at the lowest Fe concentration. The taxa that most contributed to the differences among treatments based on the SIMPER analysis were primarily larval life stages of Baetidae, two groups of chironomids (Chironomini and Tanytarsini), and Glossosomatidae (Trichoptera).

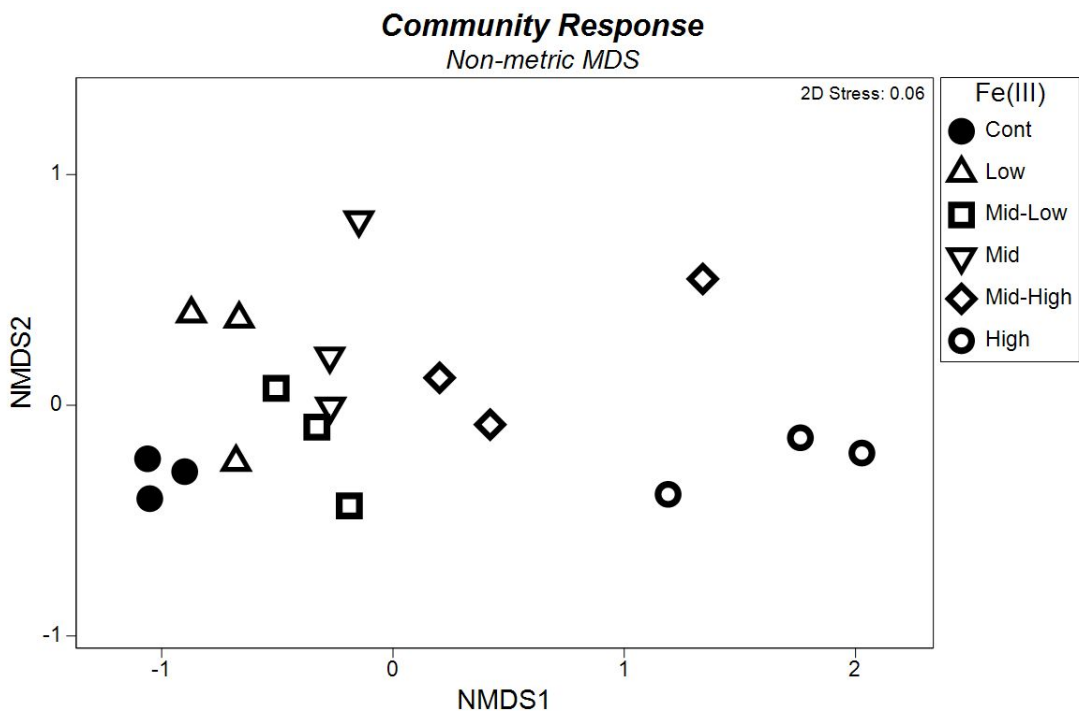


Figure 4. Non-metric multi-dimensional scaling (NMDS) plot of aquatic invertebrate community abundance in the five Fe treatments and the control. Permutational multivariate analysis of variance (PERMANOVA) was used to test for significant shifts in community composition among treatments.

Table 2. Single-factor permutational MANOVA pair-wise comparison of Fe-treated macroinvertebrate communities compared to the controls. P-values were calculated using Monte Carlo permutations. SIMPER outputs show the average dissimilarity (Distance) among the exposure levels compared to controls and the species that contributed to the upper 50% of the differences among treatments. Taxa (“L” denotes larvae; “A” denotes emerged adults) are listed from the highest to lowest contribution.

Fe(III) Treatment	p-value	Average Distance to Controls	Taxa
Low	0.072	10.60	Chironomini (L), Tanytarsini (L), Glossosomatidae (L)
Mid-Low	0.011	11.21	Simuliidae (L), Tanytarsini (L), Baetidae (L)
Mid	0.012	17.36	Chironomini (L), Tanytarsini (L), Baetidae (L), Simuliidae (A)
Mid-High	0.010	32.53	Baetidae (L), Tanytarsini (L)
High	0.002	63.09	Baetidae (L), Glossosomatidae (L)

Algal colonization was significantly altered among all dominant algal groups (**Fig. 5**). Biomass of green algae, diatoms, and total chlorophyll *a* decreased significantly across the Fe exposure gradient, with dramatic reductions at concentrations near or lower than the USEPA criterion of 1.0 mg/L (**Table S2, S4**). In contrast, biomass of cyanobacteria significantly increased as the Fe concentration was increased. These shifts in biomass of the major primary

producers were associated with significant reductions in community metabolism, likely reflecting lower biomass and primary productivity in Fe treated streams (**Fig. S4**). Among the significant algae linear models, EC20 values ranged from 0.0004 (chlorophyll *a*) to 1.935 mg/L Fe (cyanobacteria), respectively (**Table S4**).

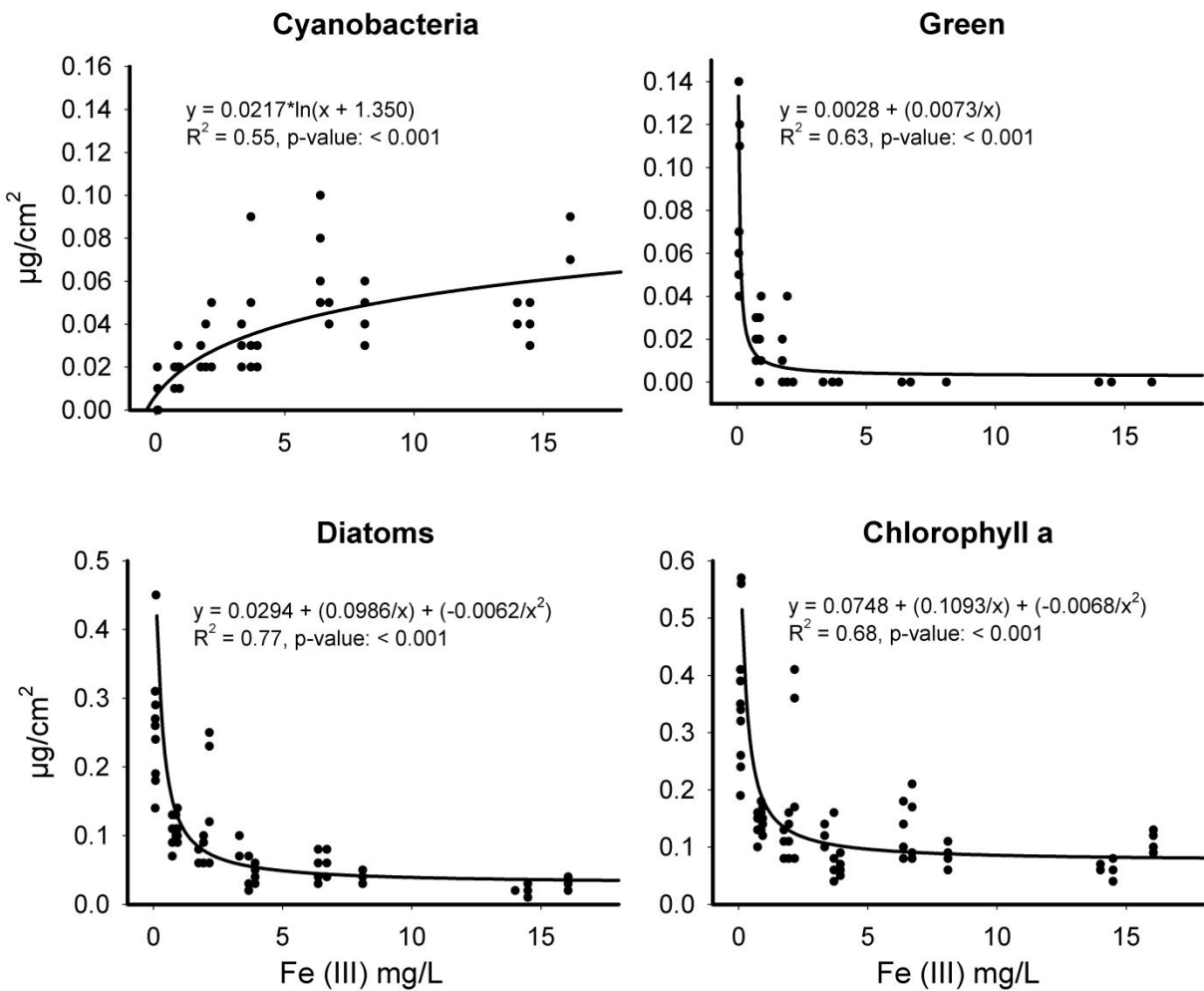


Figure 5. Effects of Fe(III) on 10 d algal colonization biomass (µg/cm²) on ceramic tiles (n=4) in stream mesocosms for major groups of the algal community.

Discussion

Ferric Fe significantly altered community composition and community metabolism after 14 d of exposure in our stream mesocosms, with effects on some endpoints occurring near or below the USEPA chronic criterion for Fe (1.0 mg/L). Abundance of both larval and adult life stages decreased for most aquatic insect taxa, and effects varied between life stages and among taxa. Larval life stages were generally more sensitive to Fe compared to emerging adults. An important exception was for Simuliidae, in which adult emergence significantly decreased, but larval abundance was unaffected. Differences in metal sensitivity among life stages of aquatic insects have been observed with field,³⁰ mesocosm,^{22, 43} and laboratory approaches.^{31,33} Although previous studies have reported decreased emergence resulting from exposure to fine sediment,^{44,45} to our knowledge, this is the first study to quantify the physical effects of Fe to benthic community emergence. Importantly, our results demonstrate that ferric Fe toxicity is life stage-dependent, and suggests that testing for Fe toxicity should incorporate multiple life stages to characterize taxa sensitivity.

Because biomass is directly related to ecosystem energetics, the effects of Fe on insect biomass is a more direct measure of subsidy export to riparian ecosystems compared to insect abundance. Given that the effects of Fe are generally considered indirect, measuring biomass is important because reduced food availability, food quality, and habitat can influence growth rates that may affect larval development and size of emerging adults.⁴⁶⁻⁴⁹ The greater sensitivity of emerging simuliid adults compared to larvae represents a comparatively greater biomass effect because adults are larger than larvae. In contrast, our observation of greater larval mortality compared to emerging adults for other groups (i.e., baetids) has implications for secondary production because larvae are unable to reach maturation. Because the duration of our study was

relatively short (14 d), it is likely that we underestimated potential effects of chronic Fe exposure to invertebrate biomass in the field.²⁶ Linked aquatic and terrestrial consumers are dependent on aquatic insect subsidies, and biomass estimates more accurately estimate subsidy dynamics compared to abundances. More research is needed to address the effects of Fe deposition on secondary production and the delivery of aquatic subsidies to terrestrial ecosystems.

We expected to see the greatest effects of Fe on sedentary taxa that were unable to avoid smothering of habitat and on grazing insects that were affected by diminished periphyton quality and biomass. Indeed, sedentary chironomids (i.e., Chironomini, Tanytarsini) and grazing caddisflies (i.e., Glossosomatidae) strongly contributed to the dissimilarity among Fe treatments. While simuliid adults were not the strongest contributors to treatment dissimilarity, we believe that high adult sensitivity was due to their sedentary pupal life stage and branched pupal gills that are susceptible to smothering.⁵⁰ Physiological traits of aquatic insects are often used to characterize metal bioaccumulation and to predict metal sensitivity in the field;⁵¹ however, biological traits such as habit (e.g., burrower, sprawler, swimmer), habitat (e.g., lotic erosional, depositional), and drift propensity may be more appropriate predictors for indirect stressors such as Fe.⁵²⁻⁵⁶

The dramatic effects of Fe on baetid mayflies were unexpected, because these organisms are strong swimmers and highly mobile,⁵⁷ and therefore should be capable of avoiding Fe precipitates deposited in the benthos. One possible explanation for the high sensitivity of baetids to Fe exposure was reduced food quantity and quality. Baetidae gut contents in low Fe treatments clearly show ferric precipitates accumulated in the gut column (**Fig. 6**). Cain et al.⁵⁸ reported that ferric Fe did not inhibit feeding of diatoms by the benthic grazer *Lymnaea stagnalis*, which suggests that periphyton with deposited Fe precipitates are not avoided. It is unclear if the dietary

363 accumulation of indigestible ferric Fe affects aquatic insect growth and survival, or if changes in
364 pH associated with the digestive tract affect exposure outcomes. More research is needed to
365 evaluate the effects of dietary accumulation of Fe.



366
367 **Figure 6:** Photo of *Baetis* sp. showing ferric Fe accumulation in gut contents. The two *Baetis* sp.
368 on the left are from control treatments, the two middle *Baetis* sp. were from low Fe treatments,
369 and the two *Baetis* sp. from the right were collected from mid-low Fe treatments. At the bottom
370 of the photo, a horizontal 0.5-mm-diameter piece of pencil lead provides a size reference.

371 Overall algal colonization after 10 d was significantly reduced in stream mesocosms.
372 Chlorophyll *a*, diatoms, and green algae biomass were among the most sensitive endpoints we
373 examined, with EC20 values well below the USEPA chronic criterion for Fe (**Table S4**). In
374 contrast, Cadmus et al.² did not observe significant reductions to algal biomass on tiles that were
375 colonized (~ 30 d) in the field before Fe(III) exposure. Because the current study examined
376 effects without prior incubation, the high sensitivity of these groups is likely due to the

functional inability of algae to initially colonize and grow. Metal oxides can prevent the attachment of algae to substrate and limit photosynthesis due to direct smothering and reduced light penetration associated with increased turbidity.^{3,5,59} However, cyanobacteria biomass showed the opposite trend in our study, with increased biomass associated with greater Fe exposure. Cyanobacteria are generally considered unpalatable and even toxic to some aquatic insect consumers, and its presence in streams is generally considered an indicator of stress.⁶⁰⁻⁶³ Cyanobacteria are also the dominant primary producers at AMD sites with neutral or higher pH.⁶⁴ The evaluation of primary producers in streams degraded by Fe(III) is important because Fe precipitates appear to affect some groups of primary producers more than others, and changes in biomass and composition affect aquatic insects that depend on these autochthonous resources.

In conclusion, we characterized the effects of ferric Fe to benthic communities by exposing naturally colonized communities under environmentally realistic habitat and exposure conditions. Importantly, we incorporated indigenous aquatic insects with complex life histories, and demonstrated that adult emergence is a particularly useful response because it characterizes the effects of a sensitive life stage and linked aquatic-derived effects to terrestrial ecosystems. Differing spatiotemporal approaches are needed to predict effects of contaminants to benthic communities using structural and functional endpoints in combination. Because traditional toxicity testing methodologies were not developed to evaluate the indirect effects of contaminants, other approaches such as mesocosm experiments are needed to characterize and predict these types of responses in real-world ecosystems. For these reasons, the development of water quality standards would generally benefit by including mesocosm testing results.

Supporting Information

Photo of stream mesocosms (**Fig. S1**); EC50 rankings for all significant structural and functional endpoints (**Fig. S2**); deposition of Fe precipitates (**Fig. S3**); community metabolism (**Fig. S4**); list of benthic macroinvertebrate taxa in control mesocosms after 14 d (**Table S1**); statistical results of linear models (**Table S2**); ANCOVA statistical results (**Table S3**); EC50 and EC20 estimates with 95% CI for significant endpoints (**Table S4**); single-factor repeated measures ANOVA statistical results (**Table S5**); PERMANOVA main effect results (**Table S6**)

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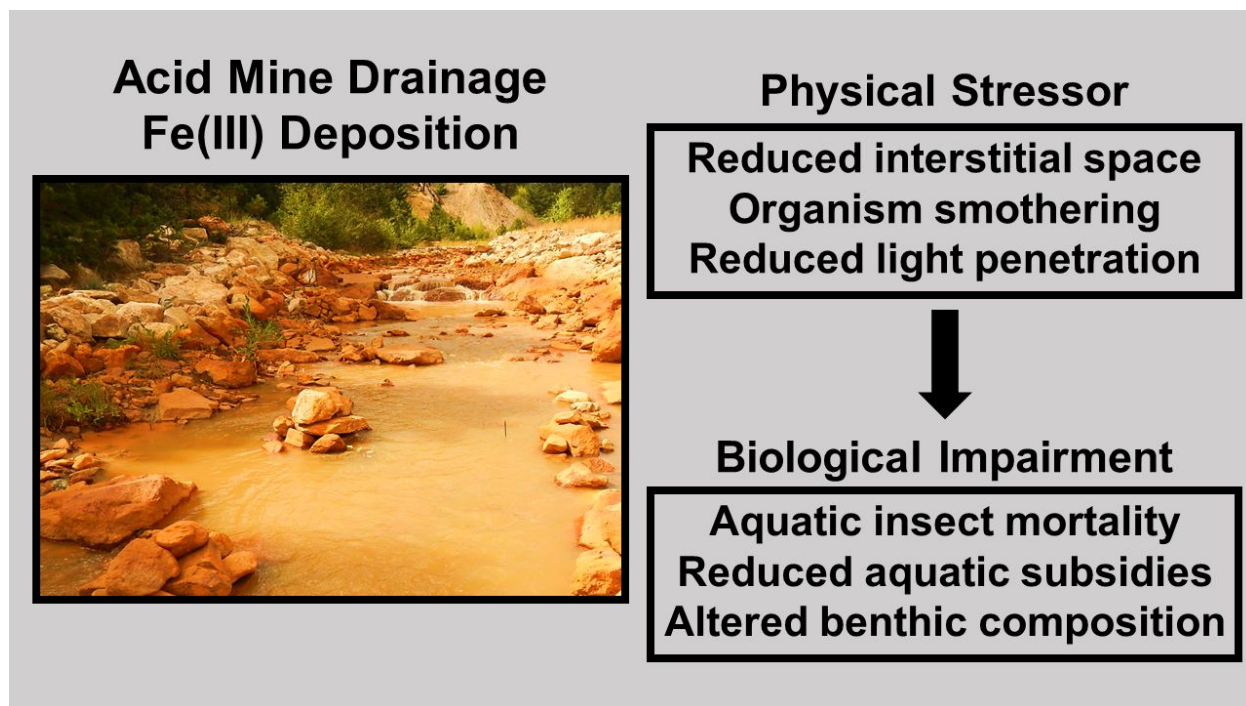
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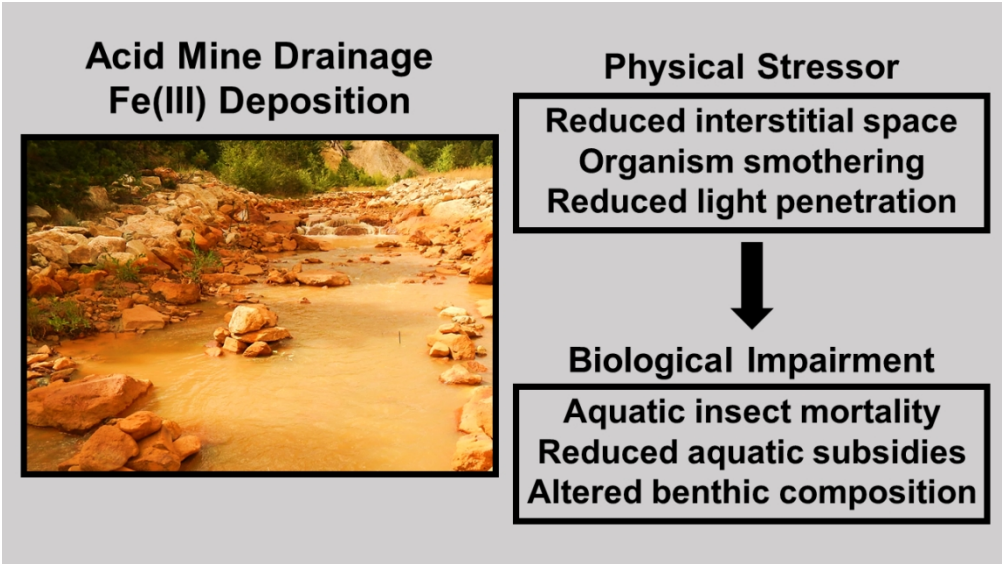


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