The Sulfate Standard to Protect Wild Rice
Study Protocol

Minnesota Pollution Control Agency

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Contents

Introduction

This document identifies key information to enhance understanding of the effects of sulfate on wild rice, and options for obtaining data that address those needs. The goal is to obtain information that would allow the Minnesota Pollution Control Agency (MPCA) to further evaluate and, if appropriate, revise Minnesota's sulfate water quality standard for the protection of wild rice. This document is intended to inform and guide the implementation of a study to further investigate the effects of elevated sulfate concentrations on wild rice. This protocol was developed in consultation with US EPA, the Minnesota Department of Natural Resources and Minnesota Tribes, and with input from interested and affected stakeholders. A meeting of scientists and technical experts was also held on May 9, 2011, to discuss a preliminary document (MPCA 2011) that presented options for testing hypotheses regarding the effects of sulfate on wild rice. This protocol is a revision of that document, reflecting the oral and written comments received from the participating scientists.

The purpose of this document is to guide the acquisition of new information as one part of the MPCA's information gathering concerning the sulfate standard for the protection of wild rice. In parallel to this new data acquisition effort, a comprehensive review of existing information will be conducted, including but not limited to: a) identification of historical and current waters supporting wild rice production, b) John Moyle's data relating water chemistry to wild rice, c) water quality data collected by the MPCA, Minnesota Tribes, and Minnesota DNR in wild rice waters, and d) a literature review of studies pertinent to this issue.

Background: Minnesota's wild-rice-based sulfate water quality standard

In its water quality standards and use classification rule, Minnesota Rules Chapter 7050, the MPCA assigns a series of use classifications to all waters of the State of Minnesota. Water use classifications, and their accompanying narrative and numeric criteria and nondegradation provisions, make up the state's set of water quality standards. Aquatic life and recreation, industrial uses, agriculture and wildlife, and domestic consumption are some of the beneficial uses these standards are intended to protect.

Minnesota's Class 4 Agriculture and Wildlife use classification covers agricultural uses (crop irrigation and livestock uses) as well as wildlife uses. Under the Class 4A use classification, Minnesota currently has a water quality standard of ***"10 mg/L sulfate - applicable to water used for the production of wild rice during periods when the rice may be susceptible to damage by high sulfate levels."*** (Minn. R. 7050.0224, subpart 2).

This 10 mg/L sulfate wild rice standard (reported as SO_4) was adopted into the MPCA water quality standards rule in 1973. Based on testimony presented at public hearings leading to the adoption of this sulfate standard, it was intended to apply both to waters with natural wild rice stands and to waters used for paddy wild rice production. The standard was based on field observations and water chemistry correlations made by John Moyle primarily in the late 1930s and early 1940s (Appendix A). Dr. Moyle was a highly respected biologist with the then Minnesota Department of Conservation, and later the Minnesota Department of Natural Resources, who concluded that "No large stands of rice occur in water having sulfate content greater than 10 ppm (parts per million), and rice generally is absent from water with more than 50 ppm." The wild rice rule was based on sound scientific evidence and, to date, the MPCA has not been presented with evidence that would support amending the rule.

The next set of wild rice-related rule amendments occurred around 1997. It was during that time that the MPCA initiated a rulemaking proceeding that led to the adoption of new rules governing water quality standards, standards implementation, and nondegradation standards for Great Lakes Initiative (GLI) pollutants in the Lake Superior Basin. This rule was codified as Minn. R. ch. 7052 and is now informally referred to as the "Lake Superior Basin" or the "GLI" rule. The 1997 rulemaking also included two major changes to Minn. R. ch. 7050: 1) A portion of the Lake Superior shoreline waters in the vicinity of the Grand Portage Indian Reservation was designated as Outstanding Resource Value Waters—Prohibited, in accordance with the provisions of Minn. R. 7050.0180. 2), Twenty-two lakes and two river segments located in the Lake Superior watershed were listed as wild rice waters (Minn. R. 7050.0470, subp. 1) and narrative language was included pertaining to wild rice under the Class 4 Agriculture and Wildlife use class (Minn. R. 7050.0224, subp. 1).

The 1997 rulemaking record reveals that originally there were 124 lake or river segments identified as wild rice waters within the 1854 Ceded Territory that were suggested for listing in Minn. R. ch. 7050 as wild rice waters. These waters were considered to be some of the more important existing and/or potential wild rice waters identified by the Fond du Lac, Bois Forte, and Grand Portage Bands. (The 1854 Ceded Territory covers much of the Arrowhead Region of Minnesota, and encompasses portions of the Lake Superior, Rainy River, and Mississippi River watersheds.) Since the provisions of the new GLI rule were specific to the Lake Superior Basin, in 1997 MPCA staff chose to limit assignment of the wild rice designation to those waters identified and agreed upon that were within the Lake Superior Basin (Minn. R. 7050.0470, subp. 1). The listing of a select number of waters as wild rice waters was intended to be part of a broader process to provide greater protection for, and greater public awareness of, the ecological importance of wild rice. These listings were also viewed as an affirmation of the MPCA's commitment to work cooperatively with Tribal governments and others concerned about wild rice waters. Inclusion of the wild rice narrative language and the rule listings were considered "first steps" toward a future statewide identification and listing of wild rice waters and the development of wild rice-related best management practices.

The 1997 rulemaking post-hearing comments noted that the 10 mg/L wild rice sulfate standard was not proposed for revision during the 1997 proceedings. In addition, the 10 mg/L sulfate standard was never intended to apply only to the 24 wild rice waters that were specifically listed in Minn. R. ch. 7050.0470. Rather this numeric standard was intended to continue to have statewide applicability to those waters used for the production of wild rice.

The MPCA is currently striving to clarify current and future implementation of the wild rice sulfate standard, which recently has come under increased questioning and contention. As part of the 2012 Water Quality Standards Triennial Review[1], the MPCA intends to clarify the definition of "water used for production of wild rice." While

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[1] The federal Clean Water Act § 303 (c) (1), requires the states, and authorized Tribes, to periodically review and amend as appropriate their water quality standards. These "triennial reviews" are intended to allow for stakeholder input and ensure recent science is incorporated into water quality standards.

revision of the 10 mg/L numeric sulfate standard continues to be evaluated for the 2012 Triennial Review, it is unlikely that sufficient data will be available to propose a revision to the numeric standard. Based on a review of available studies and information, MPCA believes that additional wild rice plant toxicity studies are needed to evaluate the effects of sulfate and other variables on wild rice, across the full life cycle of the plant, before a revision to the numeric standard can be considered.

Background: water quality standards development

Efforts by states to develop new water quality standards, or to revise existing standards, consider specific guidance provided by the US EPA based on mandates of the federal Clean Water Act. This guidance has evolved over time to reflect new understandings in the science of standards development and water quality protection.

Information about the toxicity of a chemical is one category of data used to develop a water quality standard to protect beneficial uses associated with organisms living in or consuming water. The results of toxicity tests are endpoints that describe an organism’s response following an exposure to a given concentration of a chemical for a specific length of time. The response can be based on short term “acute” endpoints or long-term “chronic” endpoints. The procedures for conducting and interpreting plant toxicity tests are not as well developed as they are for animals. The endpoints of tests using plants have been based on growth or reproduction, which are affected by chronic exposure to a chemical.

Once a toxicity threshold is identified, a water quality standard can be promulgated based on avoiding exceedance of that threshold in water bodies, and the associated impact to beneficial uses. Water quality standards are implemented for a particular body of water by specifying the *magnitude, duration*, and *frequency* of the chemical that can be found in the water and still protect beneficial uses.

To determine if a standard is attained, concentrations are monitored and compared to the concentration, or *magnitude*¸ of the standard. Monitoring also supplies two other kinds of data used to determine whether a standard has been exceeded. *Duration* describes how long the concentration of the chemical has been elevated, and *frequency* describes how often both the magnitude and duration have been exceeded. For example, most aquatic-life-based chronic water quality standards in Minnesota are implemented using a four-day average concentration of a chemical that cannot be exceeded more than once in a three-year period.

To further evaluate the current wild rice sulfate standard, information that augments the current understanding of the critical *magnitude, duration,* and *frequency* of sulfate concentration as it relates to wild rice toxicity is needed. Investigation of potential secondary effects of sulfate on the growth and reproduction of wild rice will also be important in evaluating and informing a potential revision to the sulfate standard, especially given that several current hypotheses suggest that it is not sulfate, but sulfide interactions in the sediment, that control the toxicity of sulfate to wild rice.

Purpose of this study

The goal of this study is to provide additional information to re-evaluate the wild-rice-based sulfate standard. The scientific information developed will be used by the MPCA in its decision as to whether or not a change to the existing standard is necessary, and if so, what the revised standard should be. Such a change, if warranted, would be proposed in accordance with the provisions and requirements of Minnesota's Administrative Procedure Act[2].

Considerations for evaluating the effects of sulfate on wild rice

In Minnesota surface waters, it is suspected that any negative effect of sulfate on wild rice likely involves the conversion of sulfate to sulfide—a conversion that is accomplished by anaerobic bacteria that respire sulfate instead of oxygen. During anaerobic respiration, bacterial metabolism requires that the cells donate an electron to an external chemical, such as iron, manganese, or sulfate, a chemical reaction known as *reduction*. Different groups of bacteria use different chemicals as electron acceptors. Sulfate is used as an electron acceptor by sulfate-reducing bacteria, which in principle are at an energetic disadvantage as long as certain other electron acceptors are available. In principle, electron acceptors will be consumed in the following sequence: O_2, nitrate, oxidized manganese, oxidized iron, sulfate, and CO_2. Bacteria that respire O_2 produce H_2O as a product; respiration of nitrate produces nitrite or ammonia; respiration of oxidized manganese produces reduced manganese, which can be water soluble; respiration of oxidized iron produces reduced iron, which also can be water soluble; respiration of sulfate produces sulfide; and respiration of CO_2 produces methane.

Anaerobic bacteria are most active where there is bioavailable organic matter to decompose in aquatic systems, such as decaying plants such as algae or aquatic macrophytes. The presence of water is critical, in that water is a significant barrier to atmospheric oxygen and moisture is essential for bacterial activity. Sulfide will be produced by bacteria in aquatic systems whenever bacteria consume organic matter faster than oxygen can be supplied, and after bacteria have utilized the other electron acceptors that are energetically favored.

Hydrogen sulfide is much more reactive than sulfate, and its production can alter the fundamental biogeochemical functioning of a freshwater wetland (Lamers et al. 1998, 2002; Smolders et al. 2003). In particular, in sediment porewater, hydrogen sulfide reacts with many metals, often forming insoluble precipitates. Hydrogen sulfide reacts quickly with dissolved ferrous iron, forming a variety of solid iron sulfide compounds, including pyrite. The reaction of sulfide with iron is also the primary basis for the toxicity of hydrogen sulfide to plants and animals; hydrogen sulfide binds to the iron in cytochrome c oxidase, deactivating this last enzyme in the electron transport chain (National Research Council 1979). Hydrogen sulfide, like cyanide, essentially causes chemical asphyxiation of cells by halting the respiration of oxygen. In sediments with sufficient iron, the rapid reaction of iron with hydrogen sulfide is thought to greatly reduce the potential toxicity of

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[2] Rulemaking activities in Minnesota are governed by the Administrative Procedure Act (Minn. Stat. ch. 14 and Minn. R. ch. 1400). When proposing rule amendments, the MPCA develops a Statement of Need and Reasonableness (SONAR) to document the factual information relating to a proposed rule amendment. As the name implies, the SONAR explains why a rule amendment is needed, and why it is reasonable. Public hearings are then held before an Administrative Law Judge where interested parties have an opportunity to voice support for, or opposition to, proposed amendments. This process also allows interested parties to bring forward alternative standards for consideration. Final decisions on rule amendments are made by the MPCA Citizens' Board and water quality standard revisions require approval of the U. S. Environmental Protection Agency.

hydrogen sulfide to aquatic plants that are rooted in the sediment (van der Welle et al. 2006).

The form that sulfide takes in water is pH dependent; below pH 7.0 hydrogen sulfide (H_2S, a water-soluble gas) dominates, and above pH 7.0, the bisulfide ion (HS^-), also called the hydrogen sulfide ion, dominates. The anion S^{2-}, often simply called "sulfide," is the dominant form only above pH 12, and therefore is not important to consider in this discussion. At pH 7.0, concentrations of H_2S and HS^- are equal, whereas at pH 6.0, 91 percent of sulfide is in the form of H_2S, and 9 percent as HS^-. At pH 8.0, only 9 percent is in the form of H_2S, and 91 percent as HS^- (Wang and Chapman, 1999).

It is likely that H_2S and HS^- differ in their toxicity to organisms. H_2S, being uncharged, can readily diffuse across the cell membranes of animal cells, whereas the HS^- ion may not cross membranes as readily (Wang and Chapman, 1999). Vismann (1996) found that all of the toxicity of sulfide to the commercially important marine shrimp *Crangon crangon* can be attributed to H_2S, and that HS^- did not add any additional toxicity. However, Broderius, Smith, & Lind (1977) found that some HS^- penetrated the gill epithelium of fathead minnow, adding toxicity beyond that attributable to H_2S. Apparently no studies have been published concerning the toxicity of HS^- to rooted plants, so it may be informative to assess the relative toxicity of sulfide species to wild rice.

Hydrogen sulfide produced by sulfate-reducing bacteria in organic-rich sediment in which an aquatic plant is rooted can be directly toxic to the plant, a mechanism described by Koch et al. (1990). The increased loading of sulfate has been hypothesized to alter the aquatic plant species composition of wetlands (e.g. Smolders et al. 2003, Li et al. 2009).

The production of sulfide in sediment may also have secondary and tertiary effects on aquatic plants, effects that might be mitigated or exacerbated by other biogeochemical reactions in sediment. It is difficult to know which of these reactions play a significant role in any particular setting without a great deal of supporting information.

Having described the multiple potential negative impacts of elevated sulfate concentrations, it is important to note that it is entirely possible to envision environments where sulfate has none of these negative effects. Such environments might include sites where the pH is high enough to detoxify sulfide, or where the load of labile organic matter to the sediment is insufficient to supply bacteria enough energy to reduce sulfate, or where there are sufficient non-sulfate electron acceptors that are energetically-preferred (e.g., nitrate, oxidized iron, or oxidized manganese). Indeed, Grava (1982) noted that waters with sulfate concentrations ranging from 22 to 390 ppm have been measured in commercial wild rice paddies along the Clearwater River in northwestern Minnesota.

Figure 1 and the accompanying notes present a conceptual model of potential sulfate-mediated chemical changes in sediment. Based on this model and the above considerations, MPCA staff developed Table 1 to:

1. Identify hypotheses of potential effects of elevated sulfate concentrations to shallow-water ecosystems in Minnesota,
2. Prioritize which hypotheses are most likely to be essential to further evaluating the wild rice sulfate standard, and
3. Identify study design options for evaluating the hypotheses.

It is important to note that understanding and evaluating the hypotheses of potential effects is only one key aspect of a wild rice standards study. As noted in the background section on water quality standards development, additional information is also needed about the critical magnitude, duration, and frequency of sulfate concentration (or sulfide or other associated parameters) as it relates to wild rice toxicity.

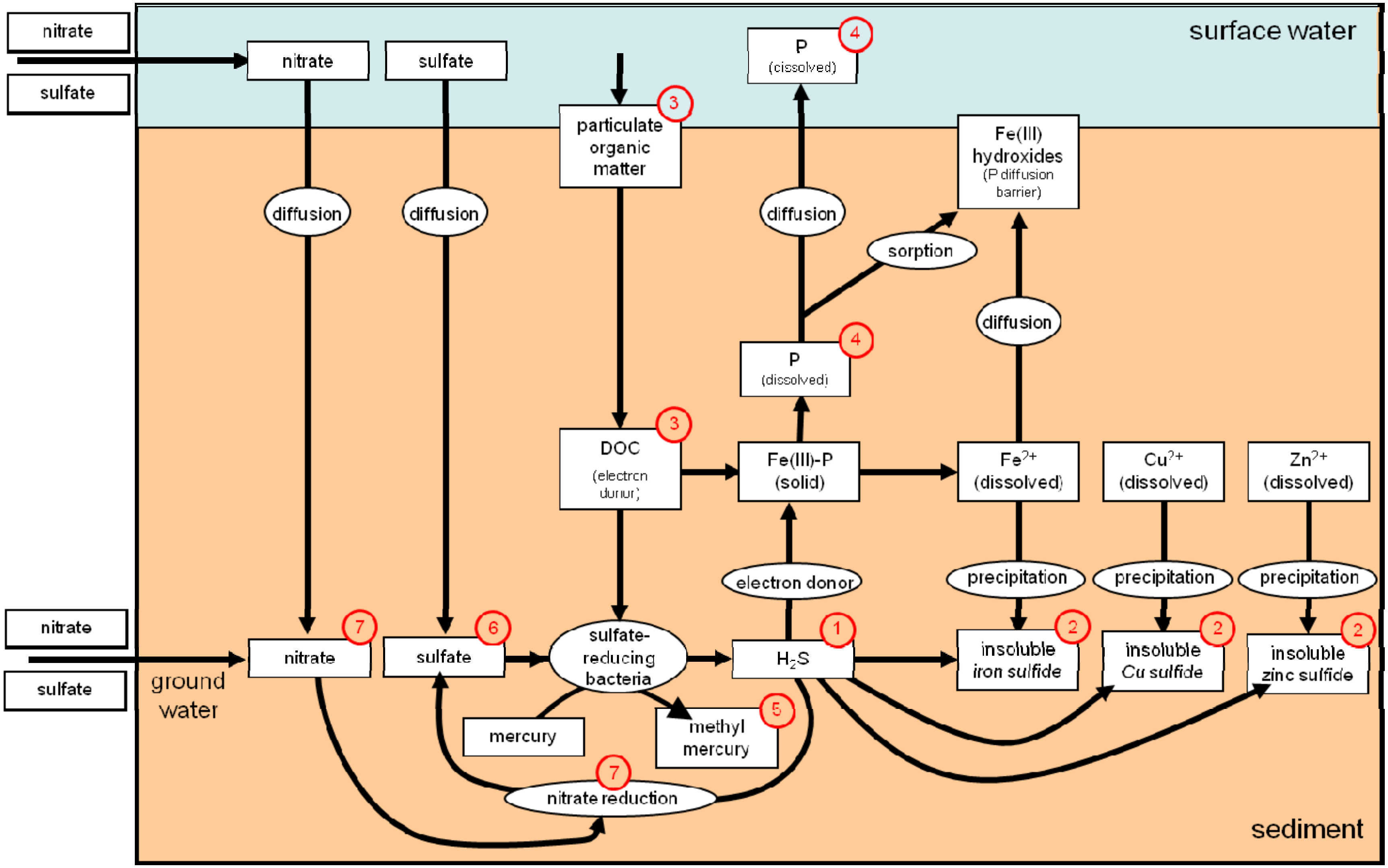

Figure 1. Conceptual model of sulfate-mediated chemical changes in sediment. This is not a complete representation of all the factors that may be controlling how sulfate might affect wild rice habitat. For instance, water movement might carry organic matter away, decreasing bacterial activity. The nitrogen cycle is much more complicated than represented. Numbered notes in the diagram are below:

Notes for Figure 1.

1. H_2S (hydrogen sulfide) is produced by sulfate-reducing bacteria, and may be directly toxic to plants and animals. However, if iron concentrations are high relative to sulfide, sulfide may be precipitated (as iron sulfide) as fast as the sulfide is produced, thereby mitigating toxicity (e.g., van der Welle et al. 2006).
2. Metal sulfides may precipitate when H_2S reacts with dissolved metals, including iron, copper, and zinc. The solid sulfides are probably less bioavailable for plant nutrition than are free metal ions, conceivably limiting plant growth due to iron, copper, or zinc limitation (these metals are essential nutrients for plant growth).
3. Organic loading controls the energy available to the system for biogeochemical reduction (electron donation, either through bacterial metabolism or inorganic chemical reaction).
4. Phosphate is mobilized and made more bioavailable when oxidized ferric iron, Fe(III), is reduced to ferrous iron (Fe(II) or Fe^{2+}), which is initially water soluble. Phosphate and iron that become dissolved under reducing conditions may then diffuse toward lower-concentration waters. Phosphate may diffuse into the surface water. Reduced iron may diffuse to the oxidized water-sediment interface, where it oxidizes and precipitates as an iron hydroxide and may intercept and hold the diffusing phosphate. Alternatively, if sulfide is available in the porewater, the reduced iron would precipitate as an insoluble iron sulfide, which does not sorb, or hold, phosphate.
5. Sulfate-reducing bacteria not only produce sulfide, but also are thought to be the primary producers of methyl mercury, the form of mercury that accumulates in fish. Methylmercury produced in the sediment can diffuse into overlying water, where it can enter the food chain by sorbing to algae and other particles that are consumed by filter-feeding invertebrates.
6. Sulfate is used as an electron acceptor by sulfate-reducing bacteria, which in principle are at an energetic disadvantage as long as certain other electron acceptors are available. In principle, electron acceptors will be consumed in the following sequence: O_2, nitrate, oxidized manganese, oxidized iron, and sulfate. The sequence may not be followed rigorously due to heterogeneity in the sediment, groundwater flow, or differing chemical reaction speeds. Naturally occurring stable isotopes of S and O have been useful in assessing the degree of sulfate reduction.
7. Nitrate is, in principle, consumed to near zero concentrations before sulfate is consumed. But if nitrate is present simultaneously with sulfide, as has been found in some systems recently, sulfide can be oxidized to sulfate. Under such conditions, H_2S may not build up even if sulfate-reducing bacteria are active. Nitrate availability can also be responsible for the oxidation of ferrous iron (Ratering and Schnell 2001)

Table 1. Hypotheses of potential effects of elevated sulfate concentrations to shallow-water ecosystems in Minnesota.

Priority* (A-C)	No.	Specific Hypothesis Regarding the Role of Sulfate	Appropriate Options for Evaluating Hypothesis						
			Indoor Lab	Outdoor Container Mesocosm	in situ Mesocosm	Intensive Field Sampling	Survey Field Sampling	Empirical Model	Mechan-istic Model
	1	**The sulfate ion itself is directly toxic to wild rice.**							
A	1A	Wild rice is only negatively affected by elevated sulfate while it is actively growing (April through August).	X	X	X				
A	1B	From September through March sufficient sulfate diffuses into sediment, or adjacent soils, so that wild rice growth is harmed.	X	X	X			X	X
	2	**The toxic agent is actually the cation associated with elevated sulfate.**							
B	2A	Elevated calcium, magnesium, potassium, or sodium correlated with sulfate is the actual toxic agent.	X	X					
	3	**Sulfide produced from sulfate is directly toxic.**							
A	3A	Sulfide produced in the sediment is toxic to wild rice seeds, reducing germination.	X	X	X			X	X
A	3B	Sulfide produced in the sediment is toxic to the roots of wild rice.	X	X	X			X	X
	4	**Sulfide produced from sulfate is sometimes not toxic to wild rice.**							
A	4A	High-iron sediments reduce toxicity of sulfide (because iron precipitates sulfide as iron sulfide, reducing the toxicity).		X	X	X		X	X
A	4B	High-nitrate systems reduce sulfide toxicity (because sulfide is oxidized back to sulfate by nitrate).		X	X				
	5	**Under certain circumstances, sulfide is not produced and wild rice grows in the presence of elevated sulfate.**							
A	5A	Relatively high nitrate availability inhibits sulfide production.		X	X			X	X
A	5B	Relatively high oxidized manganese in the sediment inhibits sulfide production.		X	X	X		X	X
A	5C	Relatively high oxidized iron in the sediment inhibits sulfide production.		X	X	X		X	X
B	5D	Water movement carries away organic matter, limiting sulfide production.		X	X	X	X	X	X
B	5E	Water movement prevents oxygen depletion in sediments, limiting sulfide production.		X	X	X	X	X	X
	6	**Sulfide indirectly reduces wild rice growth**							
A	6A	Wild rice roots develop barrier in reaction to sulfide, which inhibits uptake of essential nutrient(s) such as iron or nitrogen.		X	X				
A	6B	Sulfide precipitates essential nutrient metal(s) such as iron, copper, or zinc, making them less bioavailable.		X	X			X	X
B	6C	Sulfide production enhances P bioavailability, increasing production of organic matter, which increases sulfide production (a positive feedback loop).			X	X	X	X	
B	6D	Sulfate, through sulfide precipitation of iron, increases P bioavailability, increasing growth of perennial plants that exclude wild rice through shading, root competition, and/or allelopathy.			X	X	X	X	
	7	**External factors have increased the production of sulfide from sulfate in recent years.**							
B	7A	Reduced water movement (e.g., via impoundment) increased organic matter, increasing sulfide production (e.g., residual wild rice straw).			X	X	X	X	
B	7B	Longer ice-free period, elevated temperatures have increased bacterial activity and sulfide production.		X				X	X
B	7C	External loading of phosphorus and/or nitrogen has increased production of organic matter, increasing sulfide production.					X	X	
	8	**Wild rice is less sensitive to secondary effects of elevated sulfate when it is not actively growing.**							
A	8A	The negative effects of elevated sulfate do not persist into the wild rice growing season.			X			X	X
A	8B	Sulfate does not have negative effects September-March due to lower temperature or other reason.			X			X	X
	9	**Mercury methylation is affected by changes in sulfate concentrations.**							
C	9A	Increased sulfate enhances methylation of mercury.		X	X	X	X		
C	9B	Very high sulfate concentrations inhibit methylation of mercury.		X	X	X	X		
C	9C	Factors that remove free sulfide from pore water (4A-4B) increase the production of methyl mercury.		X	X	X			
C	9D	Factors that reduce the production of sulfide (5A-5D) also reduce production of methyl mercury.		X	X	X			
	10	**Wild rice populations in Minnesota differ in their vulnerability to elevated sulfate concentrations.**							
B	10A	Populations from habitats low in sedimentary sulfide will be most vulnerable to elevated sulfate in surface water.	X	X					

*Definitions of A, B, and C:

A: Essential to evaluating the wild rice sulfate standard.

B: Potential effects, but significantly less likely to be causative than priority A hypotheses.

C: Significant hypothesis, but does not address the wild rice issue.

Hypotheses of potential effects of elevated sulfate

From a review of the scientific literature, this document identifies numerous possible mechanisms through which elevated sulfate could have a negative effect on wild rice. Table 1 lists about 20 hypotheses as to how elevated sulfate might have negative effects on wild rice. These hypotheses can be tested through experiment, field observation, or modeling. A well-constructed protocol should result in a mass of evidence regarding the effects of sulfate. Any revision of Minnesota's sulfate standard would be based on the overall weight-of-evidence, rather than any one test.

While it is important to recognize the diversity of hypotheses, not all of the hypotheses are focused on the key objective of informing further evaluation of the wild rice sulfate standard. Each hypothesis in Table 1 is given a preliminary rating describing its priority in the design of data collection, given the study objective. Here they are sorted into three priority classifications, A, B, and C:

Priority "A" hypotheses. These hypotheses are essential to test in the evaluation of the wild rice sulfate standard.

1A *Wild rice is negatively affected by elevated sulfate while wild rice is actively growing (April through August).* It is not expected that the sulfate concentrations generally encountered in Minnesota surface water is directly toxic to wild rice, or to any other plant. Any observed negative effects of elevated sulfate on wild rice probably occur after sulfate in the water has been converted to sulfide in the sediment. However, for the sake of scientific completeness, this hypothesis is presented.

1B *From September through March sufficient sulfate diffuses into sediment, or adjacent soils, so that wild rice growth from April through August is harmed.* As in hypothesis 1A, it is not expected that sulfate is directly toxic, but rather that it is converted to sulfide, which is toxic.

3A *Sulfide produced in the sediment is toxic to wild rice seeds, reducing germination. Wild rice seeds must overwinter in the surface sediment of wild rice stands, where they might be exposed to levels of sulfide that impede successful germination.*

3B *Sulfide produced in the sediment is toxic to the roots of wild rice.* The form, or chemical species, of sulfide is dependent on the pH of the porewater. It is thought that hydrogen sulfide is the primary toxic species, a form that that is increasingly dominant with lower pH levels. After sulfide is produced by bacteria, the concentration of sulfide in porewater can be reduced by precipitation with metals or by oxidation. Plants release O_2 through their roots to oxidize and thereby detoxify sulfide in the rhizosphere. The toxicity of any produced sulfide therefore depends on multiple factors, including the pH and metal content of the porewater, the strength of the root oxidizing power, and root health as affected by nutrient availability. For example, white rice is more susceptible to sulfide toxicity under poor nutrient status due to deficiencies of potassium in particular, but also P, Ca, or Mg (Dobermann and Fairhurst 2000).

4A *High-iron sediments reduce toxicity of sulfide (because iron precipitates sulfide as iron sulfide, reducing the toxicity).* Dutch researchers have extensively investigated the role of sulfate reduction in wetlands and have documented that iron can reduce the toxicity of sulfide (van der Welle et al. 2006).

4B *High-nitrate systems reduce sulfide toxicity (because sulfide is oxidized back to sulfate by nitrate).* Research has shown that there are many microbial pathways for nitrate, including the oxidation of sulfide to sulfate (Haaijer et al. 2006, Burgin and Hamilton, 2008).

5A *Relatively high nitrate availability inhibits sulfide production.* Research in the Netherlands has demonstrated that nitrate can inhibit the production of sulfide in wetlands, even when sulfate concentrations are elevated (Lucassen et al. 2004). The practical difference between this hypothesis and the previous one (4B) might be moot, or there might be real differences that have to do with whether the sources of sulfate and nitrate are independent of each other and have similar pathways, such as shallow groundwater or surface water.

5B *Relatively high oxidized manganese in the sediment inhibits sulfide production.* In principle, oxidized manganese (and oxidized iron) will be utilized as an electron acceptor before sulfate is respired (Kirk 2004).

5C *Relatively high oxidized iron in the sediment inhibits sulfide production.* In saturated soils, oxidized iron can be the dominant electron acceptor for respiration of organic matter (e.g., Frenzel et al. 1999).

6A *Wild rice roots develop a lignified barrier in reaction to sulfide, which reduces root permeability to oxygen, water, and iron (e.g. Armstrong and Armstrong 2005).*

6B *Sulfide precipitates essential nutrient metal(s) such as iron, copper, or zinc, making them less bioavailable (Kirk 2004).*

8A *The negative effects of elevated sulfate do not persist into the wild rice growing season.* It is hypothesized that there may be potential negative effects of elevated sulfate on wild rice—elevated sulfide, for instance—but those potential negative effects are not realized because they are not retained within the wild rice stand long enough to affect the growth of wild rice. For example, sulfide developed during the winter is dissipated or detoxified before the start of the growing season.

8B *Sulfate does not have negative effects during some months, due to lower temperature or other reason.* It is hypothesized that sulfate might have negative effects during the growing season, but the negative effects do not occur during certain times of year because of environmental factors such as reduced temperature.

Priority "B" hypotheses. These hypotheses potentially occur in the Minnesota environment, but are significantly less likely to be causative than Priority A hypotheses.

2A *Elevated calcium, magnesium, potassium, or sodium correlated with elevated sulfate is the actual toxic agent, even though elevated sulfate is correlated with reduced wild rice abundance.* Correlations can be misleading. For instance, Lehman (1976) showed that experiments supporting the hypothesis that phosphate is toxic to the alga *Dinobryon* failed to control for the cations associated with phosphate; he showed that potassium was the actual toxic agent when phosphate was added as potassium phosphate.

5D *Water movement carries away organic matter, limiting sulfide production.* Because sulfide production is dependent on the availability of organic matter, removing organic matter could decrease sulfide production, just as adding organic matter may increase sulfide production and associated toxicity (e.g. Gao et al. 2003).

5E *Water movement prevents oxygen depletion in sediments, limiting sulfide production.* 5D and 5E could be difficult to separate from each other, such as water movement could provide two simultaneous, correlated, mechanisms that reduce sulfide production. On the other hand, water movement also increases the availability of sulfate to the sediment, by resupplying the boundary at the water-sediment interface as sulfate.

6C *Sulfide production enhances P bioavailability, increasing production of organic matter, which increases sulfide production (a positive feedback loop).* This positive feedback loop is well documented for the pelagic zone of eutrophic lakes and, more recently, for wetlands (Lamers et al. 1998, Geurts et al. 2009).

6D *Sulfate, through sulfide precipitation of iron, increases P bioavailability, increasing growth of perennial plants that exclude wild rice through shading, root competition, and/or allelopathy*. Competition from perennial aquatic plants (DNR 2008) has been hypothesized as a cause of decreased wild rice populations, an effect that may involve allelopathy (Quayyum et al. 1999). Whether this competition results from increased P bioavailability is speculative. Increased phytoplankton biomass could reduce light availability for wild rice during its submerged phase.

7A *Reduced water movement (e.g., via impoundment) increased organic matter loading to the sediment, reducing oxygen, and increasing sulfide production (e.g., residual wild rice straw).* Gao et al. (2003) have shown that residual straw from white rice production can cause sulfide toxicity to white rice, so it can be hypothesized that any action that decreases removal of organic matter from wild rice stands could increase sulfide production and reduced growth of wild rice.

7B *Longer ice-free period, elevated temperatures have increased bacterial activity and sulfide.* If sulfide production is limited by the temperature of surface sediment, recent increases in ambient temperature could be responsible for increased sulfide production.

7C *External loading of phosphorus and/or nitrogen has increased production of organic matter, increasing sulfide production.* If organic production has increased due to external nutrient loading, then sulfide production could have increased—independent of any change in sulfate loading.

10A *Wild rice populations from habitats low in sedimentary sulfide will be most vulnerable to elevated sulfate in surface water.* Populations of wild rice probably vary in their vulnerability to elevated sulfate concentrations. The ability of plants to accommodate environmental challenges is determined by their genetics, which usually varies within a species. It is possible that different strains of wild rice vary in their ability to tolerate elevated sulfide, given that such variance has been documented for white rice (Iimura et al. 2002).

Priority "C" hypotheses. These hypotheses concern potentially significant impacts of sulfate in the Minnesota environment, but they do not address the wild rice issue.

9A *Increased sulfate enhances methylation of mercury.* There is evidence that the addition of sulfate to low-sulfate wetlands in Minnesota enhances the methylation of mercury (Jeremiason et al. 2006), most likely by stimulating the metabolism of sulfate-reducing bacteria, which are known to methylate mercury.

9B *Very high sulfate concentrations inhibit methylation of mercury.* It has been hypothesized that high activity rates of sulfate-reducing bacteria can produce sufficient sulfide to result in a negative feedback loop in regards to mercury methylation (Benoit et al. 1999). Higher concentrations of sulfide are thought to result in reduced bioavailability of mercury to the sulfate-reducing bacteria that have the potential to methylate the mercury.

9C *Factors that remove sulfide from porewater (hypotheses 4A and 4B) increase the production of methyl mercury.* If elevated sulfide concentrations inhibit mercury methylation (Benoit et al. 1999), then it is reasonable to hypothesize that removal of sulfide might enhance the methylation of mercury.

9D *Factors that reduce the production of sulfide (hypotheses 5A and 5D) reduce the production of methyl mercury.* Inhibition of the activity of sulfate-reducing bacteria can reasonably be thought to result in reduced methyl mercury, as observed in Lake Onondaga (Todorova et al. 2009).

Methods

Introduction

It is anticipated that a) the tests described below will occur after, and be informed by, a preliminary field study that was performed in the summer and fall of 2011, and b) the preliminary field study will sample approximately 50 wild rice stands across Minnesota. The field survey will enhance the quality of the larger protocol study by identifying and characterizing field sites, obtaining preliminary data, and gaining technical experience that will make the protocol study more efficient.

Table 1 identifies the specific hypotheses that could be tested associated with the mechanism by which elevated sulfate concentrations affect wild rice in Minnesota. Each specific hypothesis is described, and appropriate options for testing that hypothesis are identified as follows:

The “Indoor Lab” option—a greenhouse or environmental growth chamber—could be used to assess to what degree varying concentrations of sulfate affect the germination and/or initial growth of wild rice seedlings under controlled conditions (e.g., Nimmo et al. 2003, Li et al. 2009).

The “Outdoor Container Mesocosm” option might be appropriate to compare the effect of varying concentrations of sulfate on the growth of wild rice plants under various defined conditions (e.g. Walker et al. 2006). When sediments are manipulated and moved into containers, it is important to provide sufficient time to stabilize sediment chemistry and to document that the sediments have chemistry similar to natural systems. For studies that run through the full life cycle of the plant, seed production, size and viability should be part of the plant metrics measured for impact. Seed production and viability can also be affected by pollination, disease and other factors that will be difficult to fully control even in tank experiments. For pollination, it may be desirable to surround study tanks with additional rice plants that serve simply as pollinators.

The “*In Situ* Mesocosm” option, while suitable for many of the hypotheses addressable in containers, may be most appropriate for assessing hypotheses requiring undisturbed sediments and ecological interactions such as interspecific competition among aquatic plants.

“Intensive Field Sampling” is an approach to collect environmental data to test hypotheses, but an approach that has less control over variables than laboratory and mesocosm experiments. It would be desirable to monitor one or more wild rice stands throughout the annual life cycle of wild rice, including under winter ice cover and during germination. A limited number of *in situ* plant growth parameters should be measured to establish a baseline for comparison with laboratory studies. Repeated sampling of selected field sites could provide useful data regarding the magnitude, duration, and frequency of water column sulfate concentrations associated with successful growth of wild rice. A key variable unrelated to sulfate concentrations is known to be water level fluctuations, which should be monitored at these sites. Other factors such as invasive species, grazing by herbivorous fish, disruption by carp, water level fluctuations, and wild rice population cycles should be considered (and measured where feasible) when selecting study sites.

“Survey Field Sampling” provides data that inform the choice of intensive field sites and representativeness of sediment and water conditions during experiments. The preliminary field survey will provide information on the range and variability of sediment and water parameters among wild rice stands. The results of the survey field sampling will serve to both constrain hypotheses and perhaps generate new hypotheses. Field data may be augmented during succeeding field seasons.

“Empirical Model” is an option to generalize the data obtained from experiments and field observations to generate an understanding of the response of wild rice to different concentrations of sulfate.

“Mechanistic Model” is an option to apply established scientific knowledge to the question of how sulfate can affect wild rice. For instance, with the data obtained during the surveys and experiments, it may be possible to apply established models of geochemical reactions that occur in sediment.

General approach/considerations for study design

The MPCA received comments from scientists and technical experts on the set of hypotheses identified above regarding the likely effects of sulfate and associated chemical, physical, and biological factors on wild rice. Based on the hypotheses and comments and the discussions at the May 9, 2011, technical meeting, a list of tasks were developed and described in general terms, laying out the basic approach and the hypotheses to be tested within the task. Those tasks/tests to be evaluated are listed below.

A. Effect of sulfate and cations on wild rice.
B. Effect of sulfide on wild rice (as seed, seedlings, and mature plants):
    1. Effect of sulfide on wild rice, keeping materials in solution with EDTA.
    2. Effect of sulfide on wild rice, allowing sulfide and metals to interact without EDTA.
    3. Effect of pH on sulfide toxicity.
    4. Effect of seed source on vulnerability of wild rice to sulfide.
C. Effect of sulfate across differing sediment types (container mesocosms).
D. Intensive field study of natural wild rice stands across the range of wild rice waters.
E. Additional field survey, expanding the 2011 Preliminary Field Study.
F. Effect of elevated sulfate, utilizing *in situ* mesocosms.

Note: The tasks described above are not listed in order of study or priority. The order that they are actually carried out will depend on a wide range of logistical consideration. In addition, the results of tasks will serve to inform and modify successive tasks.

The following paragraphs provide more details about the study design and methods, including a more in-depth discussion of each of the identified tasks.

Design of dose-response experiments

Results of dosing experiments are generally analyzed in one of two ways (Mount and Henry 2008, Cottingham et al. 2005): (1) hypothesis testing or (2) point estimation. Hypothesis testing involves statistical tests to determine which treatment means are significantly different from the control treatment. Point estimation involves regression analysis of the response curve, which is then used to estimate the concentration associated with a particular level of effect on a population. For instance "effect $\text{concentration}_{\text{percent}}$" values can be estimated; EC_{20} is the concentration estimated to affect 20% of the population based on the endpoint measured, such as growth. Specific endpoints and levels of effect will be considered further once the protocol is implemented.

In these wild rice investigations, establishing population effects will best be determined using dose-response experiments to be analyzed through regression and point estimation. The reasons for this preference are multiple: hypothesis testing is sensitive to the degree of replication within treatments and the particular exposure concentrations chosen for testing (Mount and Henry 2008, Landis and Chapman 2011). The no-observable-effect concentration (NOEC) is not necessarily a no-effect concentration—it is simply the lowest concentration that does not produce a statistically significant reduction in performance. Regression analysis provides a better means of comparing results among tests, and can be used to develop confidence intervals for point estimates. If a threshold regression model is used, a threshold exposure concentration can be identified.

While regression analysis allows more treatment concentrations in the experimental design, it is desirable to have at least three replicates at treatment concentration to ensure that it is possible to perform statistical tests between specific treatments (Cottingham et al. 2005).

In designing the dose-response experiments, the MPCA will consult existing guidance, for instance, the guidance developed by the U.S. Environmental Protection Agency (USEPA 1985). That guidance document states that a variety of data types can be used to assess adverse effects on aquatic organisms, including data on cumulative and delayed toxicity, and "reduction in survival, growth, or reproduction, or any other adverse effect that has been shown to be biologically important." (USEPA 1985, p. 28). Because procedures for conducting tests with aquatic plants, and interpreting the results of such tests, are not as well developed as for aquatic animals, the MPCA will need to consider test results that are particularly pertinent to wild rice. Other existing guidance that will be consulted includes the standardized methods that ASTM has developed (ASTM 1997, 1998, 2000), a review of nontarget plant toxicity testing (FIFRA SAP 2001), and a number of USEPA documents that are pertinent to this project (USEPA 1996a-e, 2006).

Response metrics to be measured

In dose-response experiments, the exposure effects of wild rice to the treatments will be quantified through metrics appropriate to the experiment. For instance, seed production will most likely not be measured in hydroponic experiments, since they will most likely be conducted early in the life cycle. Seed production (number and weight) will be quantified in mesocosm experiments. If *in-situ* experiments are performed, changes in biomass of wild rice will be compared to the changes in the other plant species present in the mesocosms. When practical, multiple metrics of response will be quantified, to maximize the probability that treatment effects will be detected. Possible metrics include: seed germination rate; photosynthetic rate; leaf elongation rate; chlorophyll fluorescence; live and dead biomass; biomass of leaves, shoot, and roots; number of tillers, seed weight per tiller; biomass per square meter; seeds per plant; mean seed weight; elemental concentrations in leaves and/or seeds (C, N, P, S, Fe, Zn, Cu, Mo).

In mesocosm experiments and intensive surveys, the possible response metrics are expanded, and include ecosystem-level responses (e.g., hypothesis 6D). Because sulfate enrichment may indirectly increase growth of macrophytes and phytoplankton, it may be desirable to quantify the taxonomy and biomass of competing plants, including phytoplankton. Methods for field surveys will, as much as possible, be consistent with established MPCA and Tribal monitoring methods so that data can be merged and compared. Laboratory analyses of field samples will be consistent with the methods employed in the 2011 Preliminary Field Study to the extent appropriate for producing data that can be merged and compared. The report from the Preliminary Field Study, for which an initial draft is due by January 31, 2012, will fully describe field procedures and analytical methods. A quality assurance plan will also be developed for the preliminary study, which will also specify field and analytical methods.

Porewater will be analyzed by methods developed by the MPCA after receiving recommendations contained in the report from the Preliminary Field Study, which includes a task to evaluate porewater sampling methods.

Quantification of porewater hydrogen sulfide will most likely be dependent on porewater analyses rather than *in situ* measurement with electrodes. Typical sulfide electrodes only measure S^{-2}, not HS^- and H_2S, which will be the more predominant and toxic forms in these porewaters. Because it is necessary to quantify the different species of sulfide, the most practical procedure will probably be the measurement of total dissolved sulfide, with sulfide speciation modeled from accurate measurement of porewater pH.

Rationale for hydroponic experiments and porewater analysis of sediments

Aquatic plants that grow rooted in the sediment of lakes and rivers primarily absorb dissolved essential nutrients from the water contained in the sediment via their roots, in contrast to absorbing nutrients from the overlying water via their stems and leaves (Denny 1972, Barko and Smart 1986). In aquatic sediment, the spaces between sediment particles are filled with water that is referred to as porewater. This so-called porewater contains the critical chemical environment that supplies nutrients and, on occasion, toxic levels of chemicals to plants. Soil acts as a nutrient reservoir, but the soil itself is not essential for plant growth. When the required nutrients are artificially supplied in water to the roots, soil is not required for the plant to grow. This technique, called hydroponics, is applied in plant and agricultural research allowing researchers to knowingly control and manipulate what a plant’s roots are exposed to. For instance, with hydroponics it is possible to assess the effect of sulfate on growth in the absence of sulfide, whereas when wild rice is rooted in natural sediment, it is likely that sulfate would be converted to sulfide. Most aquatic sediments contain organic matter, which supports bacterial communities that naturally can produce sulfide if sulfate is present and the sediments become sufficiently reduced. In addition, natural sediment contains nutrients of unknown initial bioavailability. Interpretation of experimental results would be made even more difficult to interpret by the progressive changed bioavailability of metals as they are either freed from the solid sediment matrix or immobilized through precipitation with sulfide, if sulfide is produced.

It is critical to gain an understanding of the chemical nature of the porewater that wild rice roots encounter in sediment. Accordingly, one of the tasks in the 2011 Preliminary Field Study is to evaluate methods to sample and analyze porewater. Porewater data may be obtained both from field sites and from experiments where wild rice grows in sediment.

Why some mechanisms surrounding sulfide need to be investigated

Several commenters on an earlier draft of this protocol suggested that it is not necessary to pursue an understanding of the chemical and biological mechanisms surrounding the transformation of sulfate into sulfide and the ultimate effect on wild rice. As noted in a previous section of this protocol, to further evaluate the wild rice sulfate standard, information that augments the current understanding of the critical *magnitude, duration,* and *frequency* of sulfate concentration as it relates to wild rice toxicity is needed. This data can be obtained through toxicity tests, via controlled laboratory and mesocosm dose-response experiments. If negative effects of sulfate are mediated through one or more environmental variables in addition to changes in sulfate concentration, information about the likely mechanisms of toxicity is needed to design dose-response experiments that will yield protective results.

It is not possible to design the most appropriate dose-response study without knowing what makes the sediment and surface water of a particular site sensitive for wild rice growing in waters elevated in sulfate. That information is necessary to determine what to specify for the study concerning variables like sediment quality (particularly pH, and concentrations of organic matter, iron, manganese, trace metals, sulfur, and nitrogen) and water quality (particularly nitrate, because it can inhibit the production of sulfide).

If mechanisms are not understood on at least a coarse level, assumptions may be made that lead to a standard that is not protective across the entire state of Minnesota, or over the full life cycle of wild rice. For example, if we assume that the mechanism behind the negative effect of sulfate is mediated through the direct exposure of wild rice roots to elevated levels of hydrogen sulfide, then dose-response studies would be designed to represent the most vulnerable aquatic habitats in Minnesota (that is, sediment with relatively high organic matter, containing porewater with relatively low pH, iron, and nitrate). However, if our assumption about the mechanism is incorrect, and the negative impact of sulfate on wild rice is in fact caused by a different mechanism, then the design of the dose-response study might be quite different. For example, according to the scientific literature on white rice, the mechanism through which sulfide reduces rice growth may be by precipitating essential trace-metal nutrients such as zinc or copper, making the metals less bioavailable—and, perhaps the concentration of sulfide that the roots are exposed to is not the controlling factor, which would be very confusing if we assume that it is. If reduced trace metal bioavailability were the mechanism though which sulfate affects wild rice growth in Minnesota, the specification of sediment for the dose-response experiments would be much different than if the mechanism is direct sulfide toxicity. In this example, a coarse understanding of mechanisms helps to decide whether to perform dose-response studies with relatively low-iron sediment or relatively low trace-metal sediment.

Seed source for experiments starting from seed

Wild rice populations probably are suited to the environmental conditions in which they grow, including both high and low sulfate concentrations of surface water. Some of the investigations in this protocol will necessarily need to obtain wild rice seeds for controlled experiments. Since wild rice seeds do not all germinate simultaneously, it will be desirable in laboratory experiments to reduce variability in growth by germinating many seeds at once and selecting sprouts from that group that have all germinated at approximately the same time.

A number of wild rice stands will be identified for possible hand-harvesting of wild rice seeds for experiments to be conducted in succeeding years, ranging from low- to high-sulfate concentrations in surface water. Most experiments will be conducted with seeds obtained from environments likely having low-sulfide porewaters, in order to provide some margin of safety in assessing the need to revise the water quality standard to protect wild rice production. It is possible different strains of wild rice vary in their ability to tolerate elevated sulfide, given that such variance has been documented for white rice (Iimura et al. 2002).

Tasks for testing hypotheses

As noted above, the MPCA has developed a series of study tasks to address the highest priority hypotheses and reflect the input from scientists and technical experts. The following tasks reflect those comments, and are described in general terms, laying out the basic approach and the hypotheses to be tested within the task. The details are to be negotiated between the MPCA and the researchers who are charged with carrying out the task, likely through a Request for Proposal process[3]. Consequently, no schedule is presented, beyond the idea that these tasks need to be conducted within two field seasons after a preliminary field study is conducted in 2011. As noted below, the results from the 2011 field study may be used in the tasks described. Some of the tasks might be repeated in the second year, modified by the knowledge gained in the first year. Depending on the results and utility of the 2011 preliminary field study, it may be appropriate to re-visit a sub-set of the field sites for more intensive data collection (Task D) or to survey additional sites beyond the initial set surveyed in 2011(Task E).

Regarding the potential for *in situ* mesocosm experiments, it is important to note that some of the participants at the May 9, 2011, technical meeting expressed skepticism that *in situ* mesocosms were worth the effort they would entail; others objected strongly to in-lake or in-stream manipulation of the environment through adjusting sulfate

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[3] The MPCA is also evaluating the potential for a "research team" approach to the study effort, which was suggested as a possible approach to this work at the May 9, 2011, technical meeting.

concentrations or other variables. Although *in situ* mesocosms probably offer the most realistic experimental setting, it was suggested that performing experiments with container mesocosms (Task C, below) might be more productive than *in situ* mesocosms. The potential negative attributes of *in situ* mesocosms include: difficulties in access, lack of nearby support facilities, low replication potential for treatments, vulnerability to water level changes and wind events, unknown groundwater flow in or out, potential for variable fertilization by birds perching on them, and logistical difficulties in installation, sampling, and maintenance. Also, any proposed *in situ* experiments would need to address concerns about manipulating natural environments. Nevertheless, *in situ* mesocosms are presented as an option below (Task F) in the event that a feasible plan is presented and resources allow. It is anticipated that any *in situ* mesocosm experiments would be installed in locations where the sediment and surface water had been characterized prior to installation; such characterization is a primary goal of the 2011 preliminary field study.

A. Effect of sulfate and cations on wild rice.

Conduct in a greenhouse or environmental growth chamber, using hydroponic methods. Test response of wild rice to varying sulfate levels and different cations using hydroponic methodology. The hydroponic nutrient solution should be similar to that observed in the sediment porewater during the 2011 field survey--probably low in sulfate and nitrate because these are often low in sediment porewaters. However, this assumes that nutrient uptake, etc. is strictly root-related, which isn't necessarily true, even though most nutrients are thought to be absorbed by the roots of aquatic macrophytes (Denny 1972, Barko and Smart 1986). Also, low nitrate in sediment can be associated with high ammonia, which can also serve as a nitrogen nutrient source, so total inorganic nitrogen should not be set too low in the hydroponic solution.

In this experiment it is important to not root wild rice in natural sediment, which contains difficult-to control and characterize quantities of sulfur, nutrients, and metals. Hydroponic methods will determine what concentrations of sulfate, metals, and nutrients the wild rice is exposed to. Participants at the May 9, 2011, technical discussion noted the importance of conducting Tasks A and B simultaneously, as Task A is not likely to demonstrate a direct effect of elevated sulfate on wild rice, and the most likely effect involves the production of sulfide. Sulfate is still important, however, as a source of sulfide. If there are logistical limitations to conducting Tasks A and B simultaneously, then the tasks should be blended together, perhaps by initially assessing a wide range of sulfide concentrations with zero-sulfide as a control, and later assessing a lower range of sulfide, again with zero-sulfide as a control.

Germinate and grow wild rice to evaluate the effect of sulfate and different cations on germination and first weeks of growth; it would probably not be practical to reach flowering stage. If practical, design a control nutrient medium that reflects the likely porewater conditions that wild rice encounters in much of Minnesota. These porewaters are likely relatively low in sulfate and nitrate. The metal content of the nutrient medium should, if practical, reflect metal data from field survey as a guide for ratios of sulfide-reactive metals Fe, Mn, Zn, Cu, Co, Ni, and Mo.

It is desirable to conduct an initial screening-level study to determine which parameters have the most potential to be controlling wild rice growth, so that the full-scale dose-response study can be kept to a manageable size.

Goal: Evaluate a number of hypotheses concerning the effect of sulfate on wild rice.

Vary: Sulfate concentrations and multiple cations (Mg, Na, K, and Ca). Add the chelator to be used in B1 as a treatment to confirm that it keeps metals bioavailable. Choose ranges of sulfate and cation concentrations based on the results of the 2011 field survey of wild rice waters. In an initial experiment, it may be possible to test the hypothesis that wild rice grows equally well in control nutrient medium compared to a nutrient medium that is high in sulfate, Mg, Na, K, and Ca. If such an initial experiment reveals no reduced growth of wild rice, it would not be valuable to conduct additional experiments in which sulfate and the cations are varied over a range.

Measure: Germination rate, wild rice survival and growth. Confirm expected concentrations of sulfate, oxygen, and sulfide.

Hypotheses addressed: 1A, 1B, 2A, 4A

B. Effect of sulfide on wild rice.

Conduct in a greenhouse or environmental growth chamber, same hydroponic methods as Task A**,** including metal concentrations. Do not again assess the cations Mg, Na, K, and Ca, as these cations are unlikely to be modified by anaerobic, sulfidic, conditions.

Sulfide exposures to the plant roots need to be maintained, even as the plants photosynthesize and the roots likely release oxygen—the goal is to isolate the roots from the atmosphere so that anaerobic conditions can be maintained. It may be necessary to use a flow-through system to maintain the target sulfide concentrations in the hydroponic growth medium.

Goal: Evaluate a) germination of seeds, including the effect of over-winter exposure to varying concentrations of sulfide, and b) growth of seedlings; determine growth-limiting sulfide concentrations and compare to other species listed in Li et al. (2009).

Measure in B experiments: wild rice survival and growth metrics, redox, sulfate, oxygen, actual sulfide concentrations (oxygen release by roots can alter the initial concentrations), pH, dissolved metal concentrations.

Subcategories of Task B:

B1. Effect of sulfide on wild rice, keeping metals in solution with a chelator.

Conduct Task B1 simultaneously with Task A, if possible, so that Task A acts as zero-sulfide control for this experiment. Otherwise, conduct Task A first, then use a sulfate concentration as a control treatment that provides adequate sulfur nutrition, as sulfur is an essential plant nutrient.

Test response of wild rice to environmentally-pertinent sulfide concentrations and porewater pH using hydroponic methodology. In this experiment it is important to not root wild rice in sediment, which contains difficult-to control and characterize quantities of organic matter, sulfur, nutrients, and metals. Hydroponic methods will determine what concentrations of sulfide species, metals, and nutrients the wild rice is exposed to. To avoid precipitation of metals with sulfide (potentially causing nutrient limitation through reducing bioavailability of sulfide-reactive metals Fe, Mn, Zn, Cu, and perhaps even Co, Ni, or Mo) include EDTA or other appropriate chelator in the hydroponic growth medium (Li, Mendelssohn, Chen, & Orem 2009).

Vary: concentration of sulfide species, in particular hydrogen sulfide.

Hypotheses addressed: 3A, 3B (sulfide is directly toxic to seeds (3A) or plants (3B)).

B2. Effect of sulfide on wild rice, allowing sulfide and metals to interact without EDTA.

Use the methods of Task B1, but omit EDTA, or other chelator, to allow sulfide and metals to interact as they might in nature. Model sulfide speciation and metal speciation and compare to measured concentrations. The reaction of sulfide with metals could produce, depending on the molar ratio of sulfide to metals, a) nutritional deficiency of precipitated metals, b) increased growth due to removal of toxic sulfide, or c) increased growth due to removal of toxic metal concentrations.

Vary: sulfide concentration.

Additional hypotheses addressed: 4A, 6B (may also address 3A, 3B).

This task may be problematic, in that the effect of sulfide would depend on the metals concentrations in the growth medium, and it may not be known what the range of concentrations is in the porewaters of wild rice beds. It may be necessary to obtain data on the metal content of porewaters prior to conducting this task. Alternatively, it may be more useful to conduct this task in mesocosms with sediment, better modeling natural conditions.

B3. Effect of seed source on vulnerability of wild rice to sulfide.

Use the methods of Task B1, but compare the sulfide sensitivity of two wild rice seed sources sampled during 2011 Field Survey. Compare: high-sulfate surface water/high-sulfide pore-water site vs. low-sulfate surface water/low-sulfide pore-water site. Alternatively, if resources do not allow a comparison of seed sources, only perform assessments with seeds obtained from low-sulfate/low-sulfide sites.

Vary: Concentration of hydrogen sulfide in porewater, by manipulating sulfide and pH.

Hypotheses addressed: Wild rice populations in Minnesota differ in their vulnerability to sulfide that is produced from elevated sulfate concentrations.

C. Effect of elevated sulfate across differing sediment types (container mesocosms)

Conduct either indoors in small pots, or in outdoor containers. Use results of 2011 preliminary field study to determine range of qualities (organic matter, iron, sulfur, etc.) of the sediment matrix, which can be manipulated through addition of materials or through the use of sediments of known composition.

Goal: Evaluate the effect of sediment types over the entire life cycle of wild rice, a critical experiment if field surveys indicate that the impact of a given sulfate concentration of overlying water is dependent on the iron or organic content of the sediment.

Vary: organic matter (perhaps vary rice straw load) in sediments of different Fe content, and vary sulfate in surface water. Measure but don't vary porewater parameters such as nitrate, ammonium, P species, Mn, Zn, Cu, and Mo unless there is preliminary evidence that the parameter may control wild rice growth or abundance, based on results from the 2011 survey or results from Task B experiments.

Measure: Wild rice survival and growth metrics over a full growing season; the vertical structure of sediment geochemistry using probes and/or porewater samplers; sediment chemistry as in the preliminary field study. Compare porewater geochemistry to findings of Task B. Measure sulfate, total and dissolved P, and nitrate in surface water. If budgets and logistical considerations allow, measure total and methyl mercury in both porewater and surface water, and consider measuring mercury bioaccumulation at the base of the aquatic food chain, in algae and invertebrates.

Hypotheses addressed: 3A, 3B, 4A, 4B, 5A, 5D, 6A, 6B, 9A, 9B, 9C, 9D

D. Intensive field study of natural wild rice stands.

Choose one or more wild rice stands for repeated observation of surface water, sediment redox state, sulfide production, and wild rice growth. Criteria for prioritizing potential wild rice sites would include accessibility for field crews, proximity to support facilities such as a field station, degree of human disturbance, and the length of record of wild rice production and environmental data. If possible, sites should be representative of the climatic and ecoregional variation across which wild rice grows in Minnesota. The multi-year cycle that many wild rice stands exhibit presents a challenge for understanding the response of a particular stand to sulfate, so special efforts will need to be made to address this issue.

Goal: To validate results obtained from container and/or *in situ* mesocosm results by comparing to the natural state of stratified biogeochemical processes, which take an unknown amount of time to reach their natural state in containers. Test to see if results from hydroponic experiments (Task B) relate to field observation of chemistry and wild rice abundance. If feasible, collect data to address ecosystem-level responses (hypothesis 6D). Because sulfate enrichment may indirectly increase growth of macrophytes and phytoplankton, it may be desirable to quantify the taxonomy and biomass of competing plants, including phytoplankton.

Measure: Wild rice endpoints. Sediment chemistry as in the preliminary field study. Compare pore-water geochemistry to findings of Task B. Compare vertical sediment geochemistry results to mesocosm sediment data. Continuously monitor redox and oxygen of overlying water to assess effects of seasonal and diurnal changes.

Hypotheses addressed: That greenhouse and mesocosm experimental results serve to support field observations. 3A, 3B, 4A, perhaps others by chance.

E. Additional field survey, expanding the 2011 Preliminary Field Study.

It may be appropriate to expand the 2011 Preliminary Field Study for a number of reasons, including: a) if it is determined that the 2011 survey did not adequately capture the full range or variability of wild rice habitats in Minnesota, and b) if additional sites need to be investigated as potential sites for intensive field study (Task D) or for *in situ* mesocosms (Task F).

F. Effect of elevated sulfate, utilizing *in situ* mesocosms.

Coordinating with any intensive field studies of stands (Task D), install in-situ mesocosms to known shallow-water wild rice habitats soon after ice-out and add sulfate to some immediately, and to some in fall, perhaps adding again under the ice. When drilling through ice, note if there is hydrogen sulfide odor.

It may be desirable to perform preliminary work to evaluate the feasibility of utilizing *in situ* mesocosms. A variety of prototype *in situ* mesocosms could be deployed to assess: a) ease of installation, b) success in isolating viable wild rice plants and other co-located rooted macrophytes, c) technology to discourage birds from perching on the mesocosm, d) viability for successfully over-wintering without mechanical disruptions.

Goal: If the mesocosms survive the winter intact, compare the effect of sulfate added all year round to the effect of sulfate addition just in the non-growing season. Compare results to laboratory and mesocosm experiments.

Vary: sulfate levels and compare year-round loading to non-growing season loading of sulfate.

Measure: Wild rice endpoints. Measure biomass of other species of macrophytes and phytoplankton. Sediment chemistry as in the preliminary field study. Compare pore-water geochemistry to findings of Task B. If budgets and logistical considerations allow, measure total and methyl mercury in both porewater and surface water, and consider measuring mercury bioaccumulation at the base of the aquatic food chain, in algae, invertebrates, and fish.

Additional hypotheses addressed: 8A, 8B (having to do with seasonality of sulfate loading). 9A, 9B, 9C, 9D (having to do with the effect of sulfate on the methylation of mercury). Test the results of Tasks B and C. Test the hypothesis that other rooted macrophytes can have positive or negative effects on wild rice.

Summary

The goal of the work described by this protocol is to obtain scientific information that will be useful in evaluating Minnesota’s water quality sulfate standard for the protection of wild rice. The current standard is valid, having been based on sound scientific evidence, and the standard continues to be enforceable. This protocol outlines the collection of data to facilitate evaluation of the current wild rice sulfate standard, inform seasonal application of effluent limits based on the standard, or lead to amending the Class 4A wild rice standard.

Much of this protocol addresses the potential negative effects of sulfide production in the rooting zone of wild rice beds. If the conceptual model presented in Figure 1 holds true, then any future revision of the sulfate standard could involve linking surface water sulfate concentrations to the production of sulfide in the rooting zone of the sediment, to ensure the surface water sulfate standard is protective of wild rice.

The study protocol describes an approach to gather useful information to explain the relationship between changing sulfate concentrations and effects on wild rice health. This approach will attempt to address the magnitude, duration and frequency of changing sulfate concentrations, along with other potential variables that may influence sediment chemistry and other conditions that could affect wild rice growth. These variables include, but are not limited to, such things as changing hydrological conditions, bacterial activity, geochemistry, plant physiology, varietal susceptibility, and interspecies competition. A collective understanding of the resulting data and these influencing factors is anticipated to provide the information necessary to define an approach for continued protection of wild rice.

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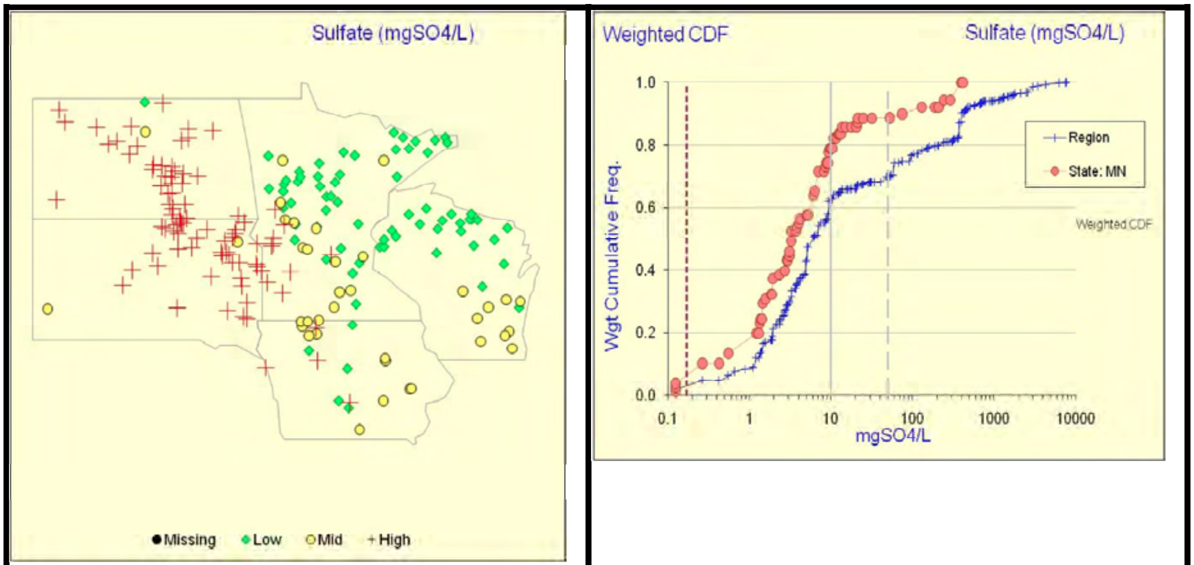
Appendix A. The environmental setting of wild rice in Minnesota

John Moyle published his observations on the correlation between wild rice occurrence and the chemistry of surface waters (Figure 3 A-C). Moyle recognized that Minnesota exhibits a strong gradient in the chemistry of surface water from soft water in the northeastern part of the state, to hard water in central Minnesota, to alkaline water in the west and southwest (Fig. 3C).

Moyle (1956) stated that wild rice is a species that requires hard water, but low sulfate concentrations: "…no large stands are known from waters where the sulphate ions exceed 10 ppm. Plantings of wild rice in the high-sulphate waters area have generally failed. The cause-and-effect relationship between sulphates and the distribution of plants is not known, but may be related to sulphur demands in plant nutrition, osmotic pressure of the water solution, or the toxicity of magnesium usually associated with sulphates."

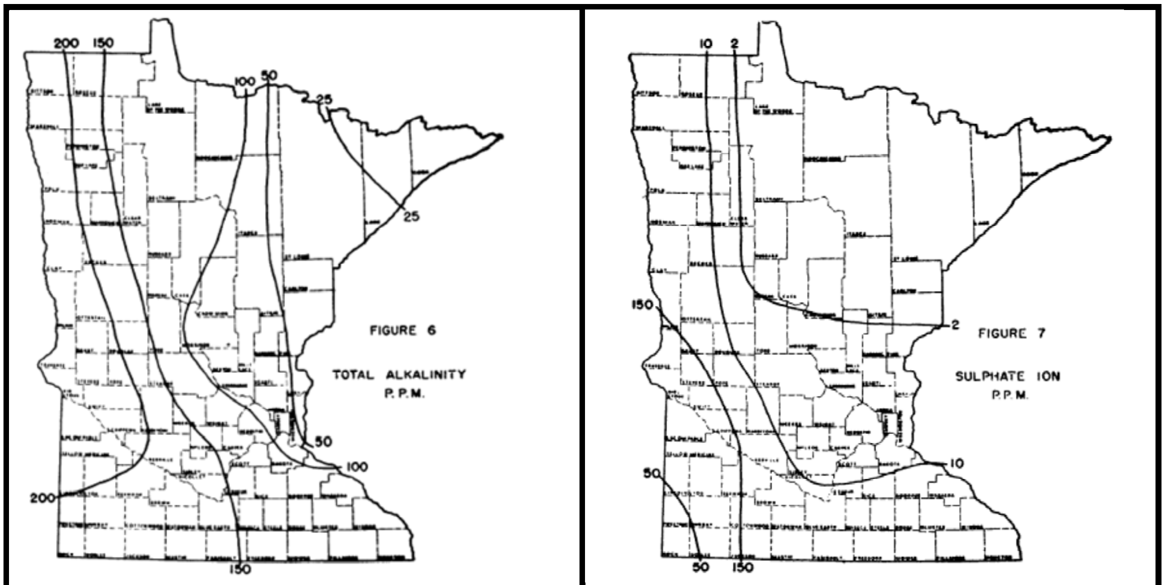
The correlation observed by Moyle between wild rice occurrence and the broad trends in the chemistry of surface water has held up over time (compare contemporary wild rice occurrence in Fig. 3D to sulfate in lakes (Fig. 2A), depressional wetlands (Fig. 4A), and groundwater (Fig. 4B). Interestingly, terrestrial crops benefit from the application of sulfur fertilizer in the low-sulfate area of central Minnesota (Fig. 4C) where wild rice stands are common, indicating that soils in that area contain low levels of sulfur.



A. Spatial distribution of sulfate concentrations in lakes. Low < 10 mg/L; Mid 10-50 mg/L; High > 50 mg/L.

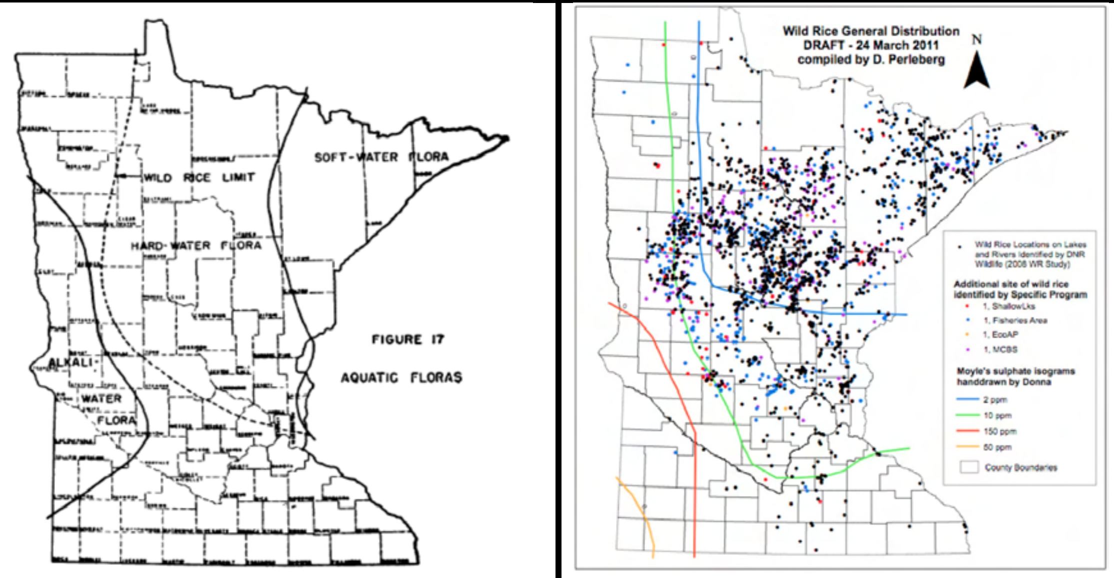
B. Frequency distribution of sulfate concentrations in Minnesota lakes.

Figure 2. Sulfate concentrations in lakes randomly selected as part of the USEPA National Lakes Assessment in 2007.



A. Alkalinity patterns in Minnesota lakes (from Moyle 1956)

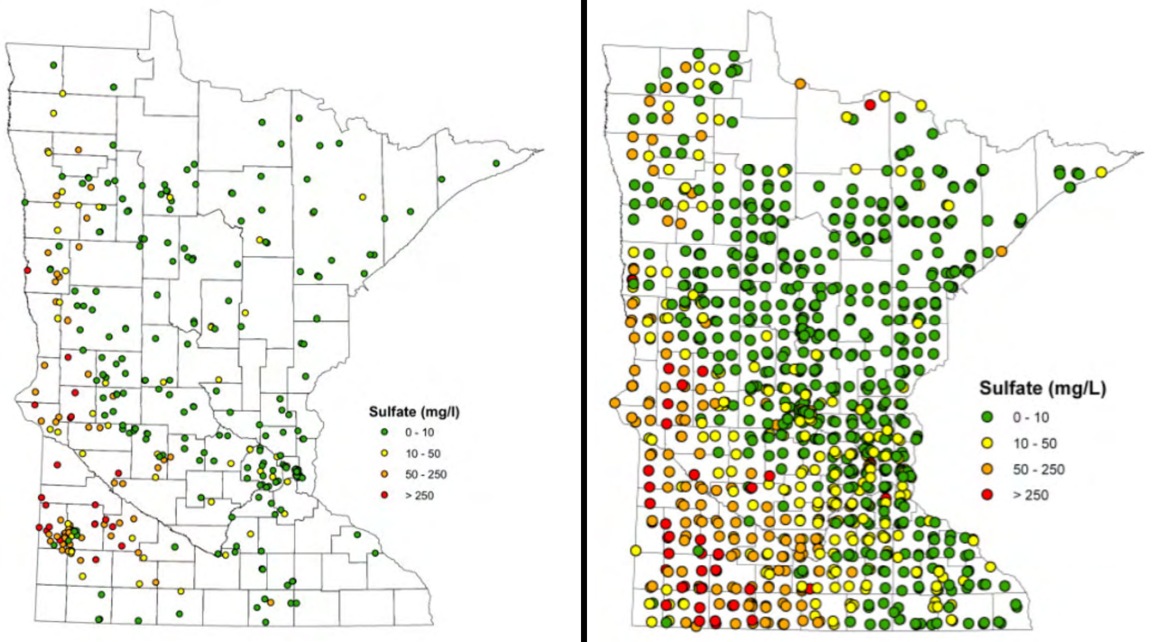
B. Sulfate patterns across Minnesota lakes (from Moyle 1956).



C. Patterns of aquatic floras across Minnesota lakes, including the western limit of wild rice, as identified by Moyle (from Moyle 1956)

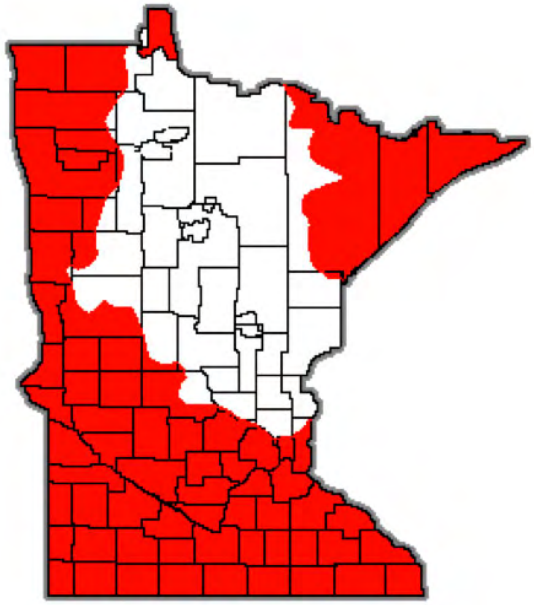
D. Contemporary pattern of wild rice distribution as documented in Minnesota DNR records (from Donna Perleberg, MDNR, 2011).

Figure 3. Patterns across Minnesota of lake chemistry (A & B) and aquatic floras (C), as published by Moyle (1956), compared to the contemporary pattern of wild rice distribution (D).



A. Sulfate concentrations in depressional wetlands (June average, MPCA data).

B. Sulfur concentrations in Minnesota groundwater (MPCA data).



C. Minnesota agricultural extension documents state that farmers can expect a positive crop response to the use of sulfur fertilizer in the non-colored areas of the state (Rehm & Schmitt, 1989).

Figure 4. Patterns of sulfate availability across Minnesota in depressional wetlands (A), groundwater (B), and soil (C).