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Abstract approved:

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Upper Klamath Lake in south central Oregon annually experiences intense blooms of cyanobacteria, primarily *Aphanizomenon flos-aquae*. Domination of the lake phytoplankton community by this single species regularly results in drastic changes to water quality. Photosynthetic activity of such extensive populations can result in pH over 10. Blooms typically expire in a short period of time, causing low oxygen conditions. Both situations are stressful to aquatic organisms and have been implicated in large scale fish die-offs, including 2 species that are federally protected. Understanding and controlling the intensity of such blooms should be an important consideration for lake management strategies.

Based primarily on observation it has been thought that both barley straw and brown marsh waters have algistatic properties. However there are numerous studies that have demonstrated colored dissolved organic matter (CDOM) or humic substances, affect algal and cyanobacterial growth in a number of ways. During the

2005, 2006 and 2007 Upper Klamath Lake *A. flos-aquae* blooms a series of controlled laboratory assays and *in situ* limno-corral experiments were conducted to asses the effectiveness of barley straw, barley straw extracts, marsh water and dried wetland plants at suppressing *A. flos-aquae* growth. Initial results of these studies indicate that the application of barley straw or dried wetland plants were most effective at suppressing and even killing *A. flos-aquae*. Marsh water and barley straw extract showed mixed results. However further analysis revealed that the degree of suppression was directly related to the concentration of CDOM present and the level of light exposure. These finding are consistent with the hypothesis that light mediated hydrogen peroxide production is the mechanism responsible for suppression of cyanobacterial growth.

Response of the Cyanobacterium *Aphanizomenon flos-aquae* to Vascular Plant Decomposition Products

by
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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Kale G. Haggard, Author

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Dr. Hayes provided materials, general guidance and editorial assistance throughout all phases of this thesis. Dr. Milligan assisted with the installation of experiments, laboratory procedures, as well as editing and revising this document. Stan Geiger provided materials, helped developed the biovolume estimation protocol, reviewed this manuscript and assisted with the design, construction and installation of experiments.

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Response of the cyanobacterium *Aphanizomenon flos-aquae* to vascular plant decomposition products

General Introduction

HABS

Events of rapid phytoplankton growth are commonly referred to as algal blooms. When planktonic photosynthetic organisms reach population densities that have negative effects on humans, animals or ecosystem function, the events are known as harmful algal blooms (HABs). HABs can also have significant negative effects on economic activity (Hallegraeff, 1993; Carmichael, 2001; Anderson et al., 2002). HABs cause harm in two principal ways. Some species of phytoplankton produce compounds that are toxic to animals and/or humans, including neurotoxins, hepatoxins and dermal toxins (Hallegraeff, 1993; Carmichael, 2001; Mankiewicz et al., 2003). Exposure to such toxins occurs either through direct contact/ ingestion of affected water or by consumption of affected fish or shellfish. Exposure can lead to a range of symptoms, ranging from mild reaction to death. Other species cause harm by modifying the environment (Hallegraeff, 1993; Anderson et al., 2002). Dense blooms, or the resultant foam and/or scum, can limit light availability to other photosynthetic aquatic organisms. This may alter the food web and community composition.

Another problem associated with dense blooms is low oxygen. This is caused by bacterial decomposition when all, or a large portion, of the phytoplankton

population dies (crashes). Low oxygen conditions are known to kill fish outright, or to render them susceptible to potentially fatal diseases (U.S. Fish and Wildlife Service, 1988; Hallegraeff, 1993; Perkins et al., 2000; National Research Council, 2004).

HABs occur in oceans, estuaries, lakes, reservoirs and rivers (Carmichael, 2001). While not a new phenomena, they appear to be increasing in frequency and distribution around the world (Hallegraeff, 1993; Roelke & Buyukates, 2001; Anderson et al., 2002). Marine HABs have received considerably more attention than freshwater HABs (434 vs. 63 citations over the past 5 years; ISI Web of Knowledge 9/10/2008). Conditions conducive to HAB formation, in both marine and freshwater systems, include an abundance of readily available nutrients and light, as well as warm water temperatures. However the dynamics of bloom formation and dispersal are not well understood (Anderson et al., 2002).

HABs are a growing and serious threat to our limited freshwater resources. Of the several classes of HAB-causing organisms, the so called “blue-green algae” or cyanobacteria (no longer considered algae), are the most common in freshwater systems (Anderson et al., 2002). These organisms cause CyanoHABs (Carmichael, 2001). Cyanobacteria produce many toxins, including hepatotoxins (which are harmful to the liver), neurotoxins (which interfere with nerve axons and synapses), cytotoxins (which damage organ tissues) and dermatotoxins (which can cause rashes and sores) (Falconer, 1999; Codd, 2000; Carmichael, 2001). The effects of long term exposure to these toxins are unknown. Published studies and reports on freshwater HABs have gone from less than 50 between 1960 and 1964

to over 900 between 2000 and 2004 (Carmichael, 2008). HABs caused by toxin-producing cyanobacteria in lakes and reservoirs used for potable water and recreation are of particular concern for human health since the primary routes of exposure are ingestion, inhalation and direct skin contact. Many areas, including Oregon, do not actively test for these compounds. (Falconer, 1999; Codd, 2000; Carmichael, 2001).

Control of HABs should be a priority for all managers of fresh water bodies. Unfortunately, few control options are currently available. There is a growing body of information demonstrating that naturally occurring dissolved organic matter (DOM) and specifically its constituents - dissolved organic carbon (DOC), colored dissolved organic matter (CDOM) and humic substances (HS) - can suppress algae and cyanobacteria (Jackson & Hecky, 1980; Perdue et al., 1981; Geiger et al., 2005). Of specific interest in this research were the effects of humic substances from wetland plants and barley straw. Humics from both sources have been shown to suppress the growth of HAB-causing algae and cyanobacteria (Jackson & Hecky, 1980; Kim & Wetzel, 1993; Barrett et al., 1996; Barrett et al., 1999; Everall & Lees, 1997; Beisner et al., 2003).

HUMICS

Humic substances, which comprise a large (50-80%) proportion of DOM, are a poorly characterized, complex mixture of organic molecules with phenolic, quinoid, alcoholic, methoxy, keto, aldehyde and carboxylic acid groups. Stable semiquinoid radicals have also been observed in HS (Paul et al., 2006). Emergent

aquatic plants are typically the largest source of organic matter in lake systems (Wetzel, 1992) and are therefore the primary source of DOM. Furthermore, Wetzel (1984) estimated that 70-80% of the dissolved organic carbon (DOC) in freshwater is composed of humic compounds. Humic and fulvic acids are the principal components of wetland DOM and marsh plants are the primary source of the DOM (Barber et al., 1999). While there has been considerable study of humics *per se*, their role in the environment is not well understood.

Phinney (1959) observed that the cyanobacteria *Aphanizomenon flos-aquae* was abundant in most areas of Upper Klamath Lake (located in Oregon, USA) but was absent in areas adjacent to wetlands. A key attribute of these wetland areas is the brown color of the water. Since Phinney's observation, there has been limited research into the effects of DOM/DOC and HS on the growth of algae and cyanobacteria. DOM/DOC/HS are currently thought to affect algal growth in three ways: (i) reducing available light, (ii) binding or chelation of essential nutrients, and (iii) toxicosis (Jones, 1992; Beisner et al., 2003; Steinberg et al., 2008).

There are no data supporting the hypothesis that DOM or HS suppress algal and cyanobacterial growth by shading. There are studies demonstrating that naturally occurring DOM in lakes reduces the amount of photosynthetically active radiation (PAR) penetrating the water column. Lindel et al. (1996) found that PAR decreased with increasing DOC concentrations. A study of 65 lakes found that the variation in light attenuation is primarily due to DOC concentration. The lake with the highest DOC concentration (23.5 g m^{-3}) also had the highest PAR

attenuation coefficient (5.21 m^{-1}). Likewise the lake with the lowest DOC concentration (0.25 g m^{-3}) had the lowest PAR attenuation coefficient (0.10 m^{-1}) (Morris et al., 1995). CDOM has also been shown to absorb the major portion of PAR. In a study of the optical properties of a single drainage basin, mean absorption of PAR by CDOM in swamps and reservoirs was 55 % +/- 28 and 32% +/- 13 respectively, compared to the mean PAR absorption by phytoplankton of was 7.4% +- 7.7 in the swamps and 12.7% +/- 7.4 in the reservoirs (Vahatalo et al., 2005). One of the many products available to suppress algal growth in ponds are dyes, which are claimed to reduce the amount of light entering the water, thus reducing photosynthesis (Aquashade, Applied Biochemists Germantown, WI).

In terms of nutrient binding, Perdue and Ritchie (2003) reported that that in many fresh water systems DOM strongly complexes with a number of elements including Fe (III), which is essential for photosynthesis. Jackson and Hecky (1980) reported a high ($r = -0.961$) negative correlation between primary productivity in lakes and reservoirs and DOC. These authors concluded that the HS component of DOM reduced primary productivity by binding iron, not by reducing light or altering pH. The growth of the cyanobacterium *Microcystis aeruginosa* showed dose-dependant suppression when treated with fulvic acid isolated from lake water, and this suppression was attributed to the availability of iron (Imai et al., 1999). Reduced growth of the cyanobacterium *Anabaena circinalis* was observed in response to treatment with a reagent grade humic acid solution, and this reduction was also attributed to reduced iron availability (Sun et al., 2005).

In the case of shading or binding of nutrients, the mechanisms of suppression are apparent. In the situation of toxicosis, the mechanism(s) are not entirely known. A mechanism hypothesized by many authors is the UV-mediated production of reactive oxygen species (ROS) (Cooper et al., 1989; Scully et al., 1996; Nakai et al., 2001; Steinberg et al., 2008). ROS are known to damage cell and organelle membranes, proteins, and DNA and can disrupt metabolic pathways. When organic matter, including HS, is irradiated, the resulting reactions are capable of producing oxidizing compounds including singlet oxygen, hydrogen peroxide, and OH^- radicals. For example, irradiated humic water controlled the growth of the green alga *Selenastrum capricornutum* (Gjessing & Kalloquist, 1991). Positive correlations between the amount of DOC in lake water and hydrogen peroxide levels are reported (Cooper et al., 1989; Scully et al., 1996). Other investigations of toxicosis revealed that phenolic compounds, particularly polyphenols, were effective in suppressing the growth of the cyanobacterium *Microcystis aeruginosa* (Nakai et al., 2001).

Although it is tempting to speculate that raising DOM, DOC, and/or HS levels could be a HAB control tool, there are reports of species and compound specificity. For example, the addition of DOM had a much greater affect on the growth of the cyanobacterium *Chroococcus minutus* than on the green alga *Desmodesmus communis* (Prokhotskaya & Steinberg, 2007). Addition of low molecular weight humic substance stimulated growth and chlorophyll a production in *Microcystis aeruginosa,* whereas addition of a high molecular weight humic

substance suppressed growth and chlorophyll a production (Kosakowska et al., 2007).

BARLEY STRAW

There have been a number of reports over the past 35 years that barley straw is effective in controlling algal growth. Much of this information comes from Great Britain, where barley straw treatments were applied to large bodies of freshwater, including lakes, potable water reservoirs, canals and streams. Suppression of annual algal blooms dominated by cyanobacteria and diatoms in several British reservoirs was attributed to the application of barley straw (Barrett et al., 1996; Barrett et al., 1999; Everall & Lees 1997). Barrett treated a 25,000 m^2 surface area reservoir repeatedly with barley straw over a 5 year period. The initial treatment was 38g straw per m^3. Nine months later straw was added at a rate of 6.5g/m^3 and six months after that another addition of 7.6g/m^3. For the following three years, 6g/m^3 were added every four months. In another test, a 140,000 m^3 reservoir was treated with barley straw at a rate of 25g/m^3. Inhibition of the filamentous green algae *Cladophora glomerata* in British canals was also been attributed to the application of barely straw (Welch et al., 1990; Caffrey & Monahan, 1999). These reports describe consistent suppression for up to six years in water bodies where blooms were formerly a regular occurrence (Barrett et al., 1999). No detrimental side effects of the barley straw treatments on aquatic and terrestrial plants and animals were reported. When barley straw was added to drinking water reservoirs,

there was no impact on water taste or odor (Everall & Lees, 1997; Barrett et al., 1999).

Several laboratory assays have shown that barley straw, or barley straw extracts, suppress various types of algae (Newman & Barrett, 1993; Ridge & Pillinger, 1996; Martin & Ridge, 1999). Ferrier et al. (2005), however, reported mixed results. For some algal species the barley straw extract suppressed growth, while it had no effect on - or even stimulated - the growth of others. Landscape scale control of algae and cyanobacteria with barley straw has not been as consistently successful in North America as in the UK (Nicholls et al., 1995; Lembi, 2001; Boylan & Morris, 2003). The reasons for this difference are not clear. Regardless, there are numerous products containing barley straw, and/or barley straw extracts, which are marketed for the control of algae and cyanobacteria in ornamental ponds and water features. A Google internet search conducted on September 16, 2008 using the key words "barley straw for algae control" gave 19,400 hits. A Google Scholar search conducted at the same time gave 1,240 hits. Clearly, science is not keeping pace with commerce. Any compound used as a pesticide, herbicide or fungicide must be registered with the United States Environmental Protection Agency before it can be used or any claims made as to its efficacy. Since barley straw has not been registered with the EPA, it cannot be legally marketed or sold in the U.S. for the prevention, removal, control or elimination of algae or cyanobacteria. In many cases these regulations are simply ignored or worked around by describing barley straw as a water "clarifier".

Other plant sources of HS have been shown to suppress algal growth, including aquatic reeds (Hong & Hu, 2007; Men et al., 2007; Nakai et al., 2006), poppies (Jancula et al., 2007), *Lantana* (a tropical shrub) (Kong et al., 2006), rice straw (Park et al., 2006a), extracts of oak (Park et al., 2006b) and "brown rotted" wood from deciduous trees (Ridge & Pillinger, 1996). Straw from other cereal crops, such as wheat, does not appear to have algistatic properties even though the straw may contain considerable amounts of phenolic compounds (Ball et al., 2001). This suggests that the kind of phenolics (e.g., there are many different structural forms of lignin) may be important, or perhaps accompanying substances affect the way the phenolics break down.

Overall, the literature supports the contention that decomposing barley straw has algistatic properties under some conditions and against certain types of algae or cyanobacteria. The mode of action is not conclusively known. Broadly speaking, the algistatic properties of barley straw are attributed to either "carbon loading" (Anhorn, 2005) or to humic substances (Jackson & Hecky, 1980; Kim & Wetzel, 1993; Barrett et al., 1996; Barrett et al., 1999; Everall & Lees, 1997; Beisner et al., 2003). The carbon loading hypothesis suggests that the addition of carbon to an ecosystem, in the form of barley straw, stimulates growth of the microbial community. Microbes, in turn, sequester the available phosphorus leading to insufficient phosphorus for cyanobacterial growth (Anhorn, 2005). The humic hypothesis states that decomposing barley straw releases a variety of substances toxic to phytoplankton. These include lignin, oxidized phenols, and methoxyphenols. Decomposing straw also releases CDOM which, in the presence

of light, undergoes a series of reactions that produce reactive oxygen species and hydrogen peroxide, compounds known to be toxic to cyanobacteria (Pillinger et al., 1994; Ridge & Pillinger, 1996; Everall & Lees, 1997; Drabkova et al., 2007).

HABs are a threat to many types of organisms and to ecosystem function. As HABs increase, they will have a much broader impact on the health of humans and the environment. Given the importance of freshwater to terrestrial life, it is imperative that low impact HAB control measures be investigated and utilized. Preliminary data showing that naturally occurring compounds such as DOC and HS can suppress the growth of some harmful algae should be substantiated. Likewise, as barley straw is the most widely used plant product for algae/cyanobacteria control, further research is necessary to the elucidate the mechanism(s) of suppression and to better target applications.

A serious of initiatives were undertaken by the Oregon State University Barley Project to assess the reputed algistatic properties of barley straw. These began with landscape level treatments of irrigation reservoirs (J.F. Schmidt and Sons nursery, Boring Oregon) and a water feature at the Oregon Garden (Silverton, Oregon). Mixed results were obtained from both tests. Some algae control was observed, but the effects were neither consistent nor absolute. The results of these tests are detailed in reports posted at http://barleyworld.org/barleystraw.php. These tests led to the general recommendation to all prospective users of barley straw for algae control that they (i) reduce the nutrient load in their water, (ii) identify the algae species present, and (iii) apply barley as part of a comprehensive program of water quality improvement. Due to the inconclusive results of these

initial tests, the experiments presented in this thesis were designed and implemented. They were directed primarily at (i) developing a controlled environment assay for measuring algistatic properties of plant materials and (ii) a more rigorous assessment of control levels at a landscape level.

Vascular Plant decomposition Products Suppress Growth of the Cyanobacterium *Aphanizomenon flos-aquae* in Upper Klamath Lake, Oregon

Introduction

Cyanobacterial harmful algal blooms (CyanoHABs) are increasing in frequency and distribution around the world (Hallegraeff, 1993; Roelke & Buyukates, 2001; Anderson *et al*., 2002; Lopez et al., 2008). CyanoHABs cause harm in two principal ways. Some species produce compounds that are toxic to animals and humans while others impact the environment by blocking light to photosynthetic organisms or causing low oxygen conditions following bloom declines. Since the 1960's Upper Klamath Lake (UKL) in Oregon has experienced nearly monospecific blooms of the cyanobacterium *Aphanizomenon flos-aquae*. In late spring and early summer, *A. flos-aquae* akinetes germinate and proceed to form macroscopic colonies or flakes of trichomes. Gas vacuoles allow *A. flos-aquae* trichomes to rise during daylight hours, resulting in a thick scum on the water surface. During blooms, lake pH rises to over 10, creating stressful conditions for many aquatic organisms. After sudden demise *A. flos-aquae* population crashes, bacterial decomposition creates low oxygen conditions (< 4 mg/L) that have contributed to die-offs of the endangered fish *Deltistes luxatus* (Lost River sucker) and *Chasmistes brevirostris* (Shortnose sucker) (U.S. Fish and Wildlife Service, 1988; Perkins et al., 2000).

UKL is a large, shallow, natural lake fed by the Williamson and Wood Rivers, as well as numerous springs. It is drained by the Link River. The drainage basin of

Upper Klamath Lake is naturally high in phosphorus with a median phosphorus content of 60 μg L^{-1} (Walker, 2001). Mass balance analysis suggests the lake has historically received nutrients at a low N: P ratio (~8:1 molar ratio), a condition that favors diazotrophic cyanobacteria such as *A. flos aquae*. However, sediment cores indicate that *A. flos-aquae* akinetes were not present in the lake prior to the 1880's (Boyd, 2002; Bradbury et al., 2004; Eilers et al., 2004). Despite low N: P nutrient ratios, diatoms and green algae were the dominant phytoplankton prior to the establishment of *A. flos-aquae* (Eilers et al., 2004).

The appearance of *A. flos-aquae* akinetes in lake sediments occurred around 1900 and coincided with the first extensive conversion of wetlands to agricultural uses. Continued development resulted in the removal of 66% of the wetland area by 1968 (Snyder & Morace, 1997; Bradbury et al., 2004; Eilers et al., 2004) (Fig. 1). The loss of these wetlands resulted in a significant reduction of dissolved organic matter input to the lake and has likely led to substantial changes in water chemistry, quality and biology (Geiger, 2001; National Research Council, 2004). Increased nutrient loading to open water due to wetland loss is likely a contributing factor to *A. flos-aquae* blooms, but there is accumulating evidence that the loss of naturally occurring plant decomposition products (dissolved organic matter, DOM) may also be an important driving factor (Jackson & Hecky, 1980; Perdue et al., 1981; Geiger et al., 2005). Emergent aquatic plants are typically the largest contributing source of organic matter to a lake system (Wetzel, 1992) and are thus the primary source of DOM. According to Phinney (1959), the contribution of DOM to UKL from marshlands would be adequate to

alter lake chemistry. A study of DOM in Hanks Marsh, one of the remaining natural marshes in UKL, found that lake DOM was largely composed of carbohydrates, lipids and proteins while marsh DOM was mainly humic and fulvic acids. Marsh plants were the dominant source of the humic substances (Barber et al., 1999). Dissolved organic carbon fractionation showed that the concentration of dissolved organic carbon (DOC) in Hanks marsh was up to twice that of the open lake (Barber et al., 1999).

Various studies demonstrate humic substances (HS) suppress cyanobacterial growth (Jackson & Hecky, 1980; Kim & Wetzel, 1993; Beisner et al,. 2003). Jackson & Hecky (1980) reported a strong negative correlation between primary productivity and increased lake organic matter content. There is preliminary and circumstantial evidence that HS affect *A. flos-aquae* populations in UKL. Phinney (1959) noted the absence of *A. flos-aquae* in UKL where humic content was high and found that humic substances stimulated the growth of green alga, but suppressed cyanobacteria in laboratory experiments. Klamath Marsh, a large (14,983 ha) wetland north east of UKL is the primary source of the Williamson River. Perdue et al., (1981) determined that fifty percent of the total organic carbon in Klamath Marsh brown waters is humic material. Flow from the marsh is seasonal, ceasing in the spring and resuming in the fall. When flowing, the river water is the color of strong tea. The absence of marsh water entering the lake corresponds with the occurrence of *A. flos-aquae,* which appears in the spring and disappears in the fall (Perdue et al., 1981). Traditionally, the recognized role of HS on aquatic population dynamics has been through a reduction in light

availability or as a complexing agent, reducing nutrient availability (Jones, 1992; Beisner et al., 2003). Recent studies have shown that HS have algistatic and algicidal effects (Steinberg et al., 2008).

Historically, HABs have been a natural phenomenon. The increase in HABs is usually attributed to cultural eutrophication (Olem, & Flock, eds. 1990; Hallegraeff, 1993; Roelke & Buyukates, 2001; Anderson et al., 2002). Reducing nutrient loads can be technically and politically challenging and as a consequence HAB control measures - herbicides, dyes, mechanical removal, increased water circulation, aeration and introduction of algae-consuming fish – provide only symptomatic relief. These solutions are often too expensive to implement on a landscape level and they do not address root causes of the HABs.

Brown water (water rich in dissolved organic matter) may play an important role in suppressing populations of some species of algae, thus preventing HABs. According to Wetzel (1984) 70-80% of the dissolved organic carbon content of freshwater is composed of humic substances. In this context, the most frequently cited and extensively studied plant material is barley straw. Suppression of annual algal blooms dominated by cyanobacteria and diatoms in several British reservoirs was attributed to applications of barley straw (Barrett et al., 1996; Everall & Lees, 1997; Barrett et al., 1999). Suppression of the filamentous green algae *Cladophora glomerata* in canals has also been attributed to barley straw applications (Welch et al., 1990; Caffrey & Monahan, 1999). Several laboratory assays have shown that barley straw, or barley straw extracts, suppress certain types of algae (Newman & Barrett, 1993; Ridge & Pillinger, 1996; Martin &

Ridge, 1999). However Ferrier et al., (2005) reported that for some algal species barley straw extract suppressed growth, while it had no effect on or even stimulated the growth of other species. Overall, the literature supports the contention that decomposing barley straw may have algistatic properties under some conditions and against certain types of algae. The mode of action has not been determined. Broadly speaking, the algistatic properties are attributed to either "carbon loading" or to "humics". UKL is unique in that barley is grown adjacent to the lake in the converted wetlands. Fields are often flooded with lake water during the winter months. The remaining water is pumped back into the lake in the spring prior to planting. This agricultural practice may add significant volumes of brown water, which might have an effect on UKL *A. flos-aquae*.

The objective of this research was to test the hypothesis that the loss of wetlands is a contributing factor of *A. flos-aquae* HABs in UKL by assaying the effects of wetland and terrestrial plant (barley straw) DOM on the growth of *A. flos-aquae*. Positive results of aquatic plant DOM suppressing *A. flos-aquae* would demonstrate additional ecosystem services of wetlands and would provide incentives for wetland conservation, rehabilitation and reestablishment. Demonstration that barley straw is an effective agent for suppressing *A. flos-aquae* could provide a role for production agriculture to take part in improving ecosystem quality.

Materials and methods

Multiple experiments were conducting using various treatments applied to mesocosms and limnocorrals. These experiments are described in Table 1. For the mesocosm assays, the experimental units consisted of 3.8 L glass jars. The mesocosm experiments were conducted under greenhouse and outdoor conditions. The limnocorrals allowed us to conduct experiments *in situ* in UKL by isolating columns of lake water.

Barley Source Material

Barley straw used in the first two mesocosm assays was a composite of breeding lines grown at the Oregon State University Hyslop research farm in 2003. Barley grown in this area is subject to high disease pressure. When exposed to pathogens, plants can respond by producing antimicrobial compounds (Castro & Fontes, 2005). This was a cause for concern, in that barley exposed to high disease pressure may have high levels of such compounds, which may increase the efficacy of this barley straw in suppressing cyanobacteria. For all the subsequent assays, barley straw (variety Baronesse) was obtained from Klamath Basin farms. For all mesocosm experiments, barley straw was cut into 1~2 cm pieces and added to each experimental unit. For limnocorral assays barley straw was loosely stuffed into plastic mesh bags and attached to the top crossbar of the barley straw treated limnocorrals.

Barley straw infusion

The initial barley straw infusion was made by placing 175 g of dry barley straw in 25 L de-chlorinated tap water. The barley straw was a composite of breeding lines grown at the Oregon State University Corvallis, Oregon Hyslop research farm in 2003. This solution was continuously aerated and kept under greenhouse conditions. Fourteen hours of supplemental illumination per twenty four hour were provided by Sun System III 400 watt high pressure sodium lamps (Sunlight Supply Inc., Vancouver, Washington USA). Midday light intensity, measured at solution level averaged 500 μ mol m/s. Day and night temperatures were 21 +/- 5^0 C and 16 +/- 5^0 C. After 60 days, the straw was removed and drained then de-chlorinated tap water added to bring the volume back to 25 L. For the subsequent assays, barley straw infusion was made by adding 100 g of Klamath basin barley straw to 10 L of de-chlorinated water. The solution was kept in a greenhouse at Corvallis, Oregon (as described previously) and continually aerated for four weeks, after which the straw was removed, drained and de-chlorinated water added to bring the volume back to 10L.

Barley Field Water

Barley field water was from a field on the north side of Coon Point, adjacent to Hanks Marsh. Barley was harvested from this field in the summer of 2006 and the field was flooded over the fall winter, and spring. While the water was being pumped off in preparation for planting, samples were collected at the outflow and were kept refrigerated until used.

Marsh Plant Source Material

Wetland plants were harvested in 2006 from Hanks Marsh, UKL and dried in a plant material dryer for 5 days at 41° C then stored in plastic bags until used. The dried wetland plants consisted of approximately 75% Rush (*Scirpus acutus*) and 25% cattail (*Typha latifolia*). The dried wetland plant material was cut into 1 ~ 2 cm pieces which were added to the experimental units.

Marsh Water

Marsh waters from Caledonia and Klamath Marsh were collected either via pump or bucket and transported to experimental sites in polyethylene containers. Klamath marsh water was collected from two sites, mile post 6 on the Silver Lake Road (42º 53' 20.14" N, 121º 42' 44.22" W) or Kirks Bridge, Forest Road 43 (42º 44' 29.5" N, 121º 50' 2.79" W).

Field and Laboratory Measurements

Mesocosm Assays

Mesocosm assays were performed during the summer months from 2005 to 2007. Mesocosm studies were performed either in a greenhouse at Oregon State University Corvallis OR, or outdoors at the Klamath Basin Research and Extension Center, Klamath Falls OR. For mesocosm assays preformed in the greenhouse, light and temperature conditions were as described under "Barley Straw Infusion". Due to absorbtion by the glass roof and attenuation by neutral density screening UV B radiation (280 -320nm) was greatly reduced (0.96

$\mu W/cm^2$; PMA2100 meter and PMA2102 sensor, Solar Light Company, Glenside, Pennsylvania, USA) in greenhouse treatments. Mesocosms were kept at 20° C using a PolyScience 5705P recirculation / chiller (PolyScience, Niles, Illinois USA).

The mesocosm assay conducted outdoors at the Klamath Experiment Station was designed to more closely approximate lake conditions such as temperature, elevation and light. This facility is located 12 km south of UKL at an altitude of 1066 m. Average UV radiation was 22.7 $\mu W/cm^2$ or 23 times greater than greenhouse studies. To test for the possible effect of shading by the addition of barley straw, a shade treatment was included in the experiment. The shade treatment consisted of a neutral density filter that reduced light to levels comparable to the straw treatment (150-200 μmol quanta m^{-2} s^{-1}). Temperature was controlled by placing the mesocosms in 1.2 m x 1.5 m tanks with continually circulating UKL water from an adjoining canal. Water temperature averaged 25° C, which is 1 - 2° C higher than water in the lake.

For all experiments, mesocosms were sampled for *A. flos-aquae* biovolume, abundance of other algal species and phosphate concentration. Mesocosms were gently homogenized prior to sampling. Dissolved soluble reactive phosphate samples were filtered using a syringe filter (GF/F) and stored frozen in polypropylene centrifuge tubes until processed. Samples for biovolume were fixed with Lugols solution (1% final concentration) and stored in polypropylene centrifuge tubes in the dark until analyzed. Temperature, pH and specific

conductivity were monitored at each sampling interval with a Hydrolab Surveyor (Hach Environmental, Loveland, Colorado USA).

Due to the difficulty in maintaining *A. flos-aquae* in culture, it was collected directly from Upper Klamath Lake prior to the start of each assay. Sufficient volume for each assay (50~100 L) of lake water was collected from a depth of 0.25 m, approximately 30 m offshore of the Skillet Handle. For each mesocosm assay, 1 L water samples were diluted with 500 ml of filtered lake water using a Whatman GF/F filter (Whatman International Ltd, Maidstone, England). For controls, de-ionized water was added to bring the total volume to 2.5 L. For treatments, de-ionized water was mixed with extracts or dry plant material. Each mesocosm was treated with 1.5 ml of the insecticide Sevin (1.25 ug L^{-1} Naphthyl N-Methylcarbamate) (Sevin, GardenTech, Lexington, Kentucky USA) to preclude grazing by zooplankton (Relyea, 2006). This concentration effectively killed all cladoceran grazers (data not shown) and was five-fold below the lowest observed effect concentration for *A. flos-aquae* (Ma et al., 2006). Aeration and circulation were provided by micro bubble air diffusers powered by aquarium pumps. For each experiment, the treatments were arranged in a replicated randomized block designs.

Limnocorral Assays

The *in situ* experiments were conducted during the summers of 2006 and 2007 (Table 1). Limnocorrals were constructed of PVC pipe frames 1.3 m in diameter and 2.6 m in length, for a total volume of approximately 3000L. The frames were

placed inside heavy duty polyethylene tubes equipped with closed cell foam collars for flotation and either a chain anchor (for open bottom enclosures) or a sand-filled 3.8 L jug (for closed-bottom enclosures). Treatments were replicated 3 and arranged in random block design. Limnocorrals were placed in an embayment protected from dominant northerly winds adjacent to the Skillet handle. Rhodamine dye was added to a randomly selected unit in order to determine the rate of water exchange/treatment loss. The 2007 limnocorral assay was installed at the onset of a lake-wide crash of *A. flos-aquae.* As such the assay was unsuccessful and no results are reported.

A depth integrated sampler (2.5 m long PVC pipe with a remotely operated valve on one end) was used to remove a column of water from the top 2 m of each limnocorral. The sample was then drained into a bucket, homogenized, and subsamples removed with a 50 ml syringe. Each limnocorral was sampled before the addition of treatments, then daily through the duration of each experiment for *A. flos-aquae* biovolume and other algal species abundance and every other day for rhodamine and phosphate concentrations. Limnocorral dissolved oxygen, pH, temperature and specific conductivity were monitored daily at a depth of 1.5m depth using a Hydrolab Surveyor.

Laboratory analysis

A. flos-aquae biovolume was determined by counting and measuring individual units using an inverted microscope at 200X magnification and an Utermohl counting chamber (2 ml volume). *A. flos-aquae* density was determined

by counting all units in a randomly selected field of view until 400 filaments were counted, or 20 fields of view examined. Average *A. flos-aquae* length was determined based on the measured length of 40 units in transects. *A. flos-aquae* biovolume for each sample was calculated according to the equation:

$$Biovolume = N \bullet \overline{L} \bullet D \bullet V^{-1}$$

where N is the number of *A. flos-aquae* units. $\overline{L}$ is the average length of a unit, D is the diameter (5 μm) and V the volume analyzed. Diameter of *A. flos-aquae* was determined using a calibrated ocular micrometer.

A. flos-aquae growth was calculated according to the equation:

$$\mu = \ln (B_1 \cdot B_0^{-1}) \cdot (t_1 - t_0)^{-1}$$

where μ is the intrinsic rate of growth in units of per day; B_0 is the biovolume value at day zero (t_0) and B_1 is the biovolume value at day one (t_1).

A. flos-aquae growth rates were then compared using the Tukey Kramer multiple comparisons test ($\alpha = 0.05$). Comparisons were made with in treatments (for each assay) as well as across treatments (all assays).

Phosphate concentration was determined by the ascorbic acid colorimetric method (Greenberg et al., eds. 1985). Water temperature, pH, specific conductivity and dissolved oxygen level were monitored throughout each assay with a Hydrolab Surveyor. Rhodamine dye samples were measured with a Turner Designs 10AU fluorometer (Turner Designs, Sunnyvale, California USA) fitted with rhodamine excitation and emission filter set (10-0419).

Results

Exposure to barley straw caused a dose-dependent decline in *A. flos-aquae* growth (Fig. 2). Addition of barley straw at 5 g / L caused rapid loss of biomass within 24 hours. A reduction in light using neutral density screening to 270 μmol $m^{-2}s^{-1}$ (the level of the 5 g / L barley straw treatment) had no significant effect on *A. flos-aquae* growth (Fig. 2). In general barley straw additions caused rapid mortality of *A. flos-aquae* (Fig. 3 and 4) except on one occasion where *A. flos-aquae* growth was repressed (Assay 3, Fig. 3b). In the assay where barley straw did not cause mortality, the addition of wetland plant material at the same rate resulted in rapid AFA mortality (Assay 3, Fig. 3b). Results of barley straw extract on *A. flos-aquae* growth were mixed. A dose of 50 ml / L (assay 1) had no significant impact; where as a dose of 250 ml / L (assay 3) caused mortality (Fig. 3). Both the barley field and marsh water treatments had no effect on *A. flos-aquae* growth, except in the limnocorral assay where growth was repressed by the addition of Klamath Marsh water (Fig. 3 and 4).

The addition of barley straw significantly altered water chemistry compared to the control. In both the mesocosm and limnocorral assays phosphate ranged between 4-8 mol P/g barley straw. At the initiation of the assay the average percent oxygen saturation ranged of the limnocorrals was between 70 to 80 %, and pH was between 8.5 and 9. By the second day the percent oxygen saturation in the barley straw treatment dropped to 0.9% and the pH to 7.3, whereas the control percent oxygen saturation and pH remained about the same.

Although dose responses were not linear when compared together, two distinct patterns based on location are apparent (Fig. 5). Assays conducted in or around UKL had 23-fold greater UV exposure and a 3 fold greater impact on *A. flos-aquae* mortality for a given dose than those conducted at the Corvallis site (Fig. 5). For each location treatment efficacy was linearly related to the concentration of CDOM, based on absorbance measurements between 300 – 500 nm (Fig. 5).

Discussion

Both barley straw and wetland plant material can suppress or kill *A. flos-aquae*. While we are unable to definitively explain why this occurs based on these assays, we did find that efficacy is codependent on CDOM concentration and the level of UV radiation exposure. CDOM is reported to affect algal growth in three ways: through shading, binding or chelation of essential nutrients and toxicosis (Jones, 1992; Beisner et al., 2003; Steinberg et al., 2008). Shading was not a factor in our experiments. When we reduced ambient light by 80% using neutral density filters, there was no reduction in growth of *A. flos-aquae*. The wetland plant treatment was the most effective in suppressing *A. flos-aquae* growth. It also released more CDOM on a dry weight basis than barley straw (Fig. 6). Barley straw was most effective at suppressing *A. flos-aquae* in the presence of high UV radiation. Both these treatments resulted in near complete *A. flos-aquae* mortality within 24 hours. Because of the rapidity of the response, these results are consistent with toxicosis and not the binding of essential nutrients. Mortality due to binding of essential nutrients can be prevented in the short term by remobilization of the compound from cellular reserves. It has been demonstrated that compounds such a phenols leach from plant material and are toxic to cyanobacteria (Nakai et al., 2001; Waybright et al., 2008). Other compounds toxic to cyanobacteria are formed when CDOM is degraded by UV. The best characterized reaction is the UV-mediated formation of hydrogen peroxide.

UV-mediated CDOM degradation occurs through a series of photochemical reactions producing singlet oxygen, peroxy radicals, hydroxyl radicals, superoxide, hydrogen peroxide and humic-substance organic radicals (Cooper et al., 1989; Scully et al., 1996). These radical species eventually produce hydrogen peroxide and concentrations can reach 1 – 2 μM in natural waters (Scully et al., 1996). In addition, stable semiquinoid radicals have been identified and oxidized phenols, especially methoxyphenols are known to be highly toxic (Pillinger et al., 1994; Everall & Lees, 1997; Steinberg et al., 2008). Accumulation rates of hydrogen peroxide are positively correlated with colored dissolved organic carbon concentrations (carbon that absorbs light between 300 – 500 nm) (Cooper et al., 1989, Scully et al., 1996). Through Fenton reactions, iron (III) consumes some peroxide to form highly reactive hydroxyl radicals. All organisms are exposed to oxidative stress and as such have developed reactive oxygen scavenging mechanisms, such as superoxide dismutase, catalase, ascorbate oxidase and peroxidase. However when reactive oxygen species (ROS) production exceeds the capacity of these scavenging mechanisms, membrane integrity is compromised, DNA and protein are also damaged. Injury to mitochondrial chloroplastic membranes is particularly dangerous as it interrupts electron transport resulting in additional ROS production. These effects lead to necrotic cell death or induce programmed cell death (Bidle & Falkowski, 2004). Our results are consistent with these findings. When either the concentration of CDOM or the level of UV radiation was increased, the result was more suppression of *A. flos-aquae* growth.

Marsh water was inconsistent in suppressing *A. flos-aquae*. The dose rate of marsh water treatment which suppressed *A. flos-aquae* growth was 80 ml / L compared to rates of 250 ml /L which had no effect.

While barley straw was quite effective in suppressing or causing mortality of *A. flos-aquae*, the dosage rates we used did have potentially serious impacts on water phosphate and dissolved oxygen levels. However, for the assays conducted in and near the lake (high UV), a lowest effective dose has not been determined. The possibility remains that a lower dose of barley straw may be effective in suppressing the growth of *A. flos-aquae* without negative impacts on water quality. Barley field water, which had the lowest CDOM concentration, simply had no effect on *A. flos-aquae* growth. Without knowing the lowest effective dose of barley straw on *A. flos-aquae* in UKL, it is difficult to make a recommendation as to the utility of barley straw for HAB control at this location.

This work does support the idea that wetland loss in UKL could be a contributing factor to *A. flos-aquae* HABs due to reduced input of CDOM from the wetlands into the lake. This role of wetlands in maintaining water quality merits further research. As converted wetlands are reconnected to UKL, the impact they have on *A. flos-aquae* can be quantified via a coupled remote sensing and *in situ* monitoring program.

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Figure 1. Relationship between the relative abundance of *A. flos-aquae* akinetes and the percent of wetlands lost from UKL over time (from Geiger, 2001 and Eilers et al., 2004)

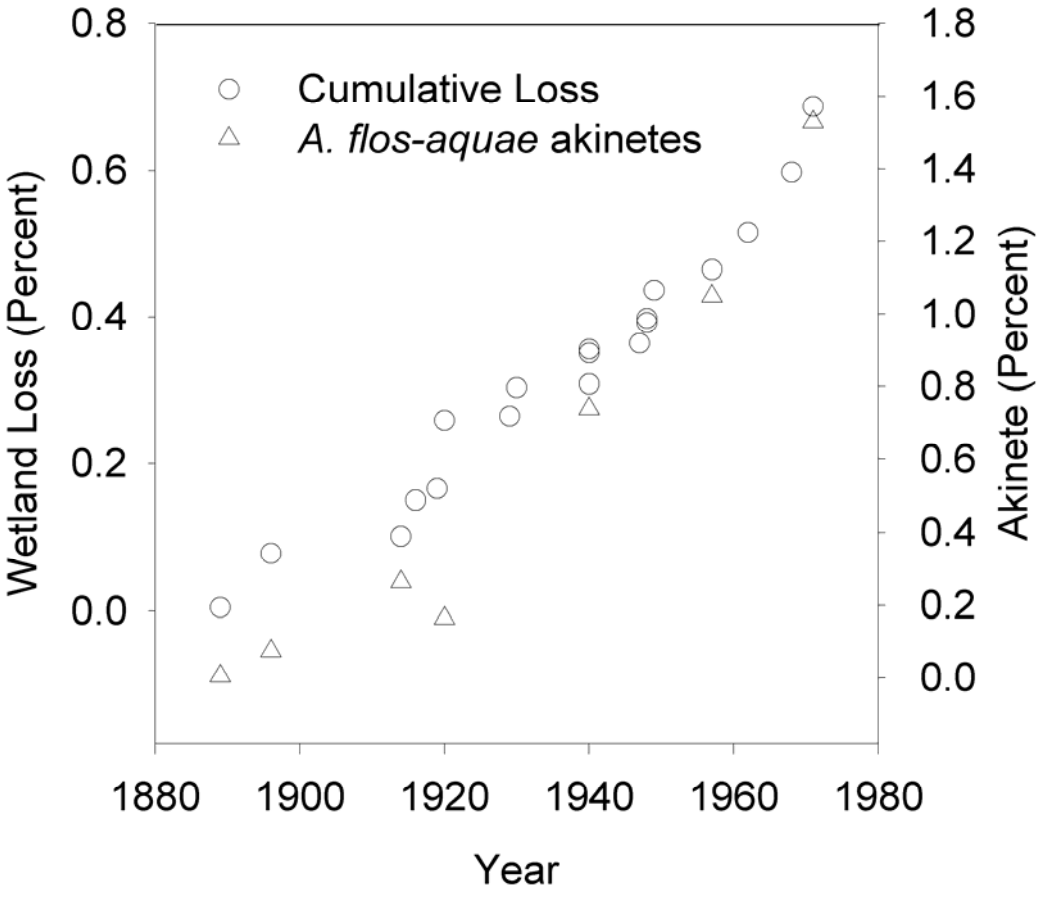


Figure 2. Average *A. flos-aquae* biovolume of the control, screen, 1 g / L, and 5 g / L barley straw treatments in mesocosm assay 3. Mean ± standard error, n=4 for all treatments.

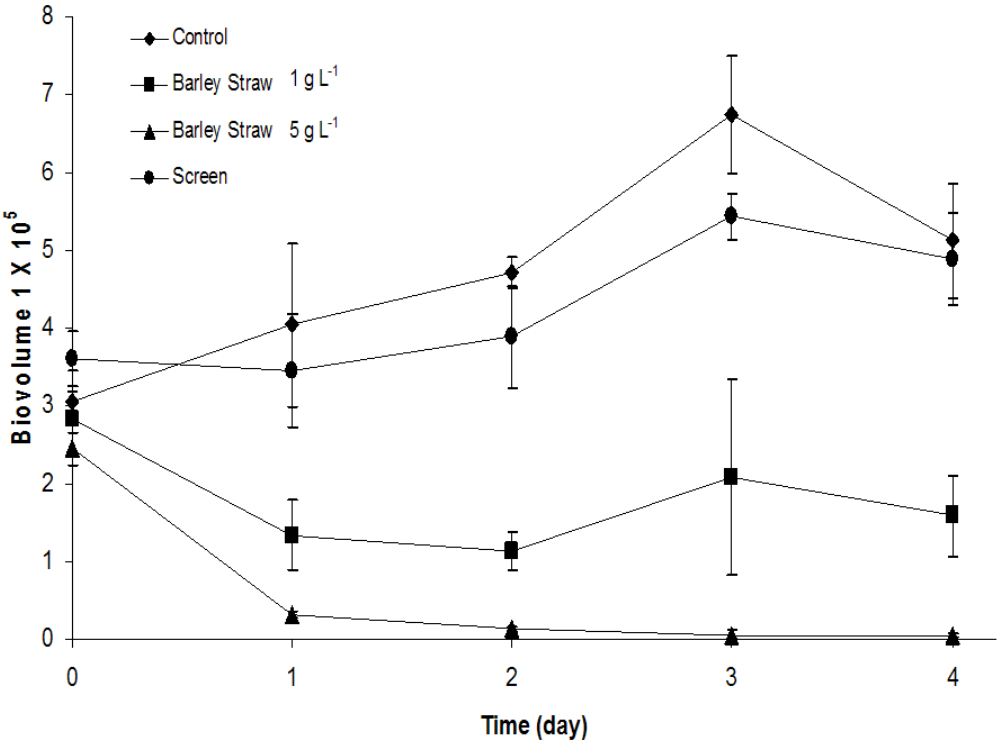
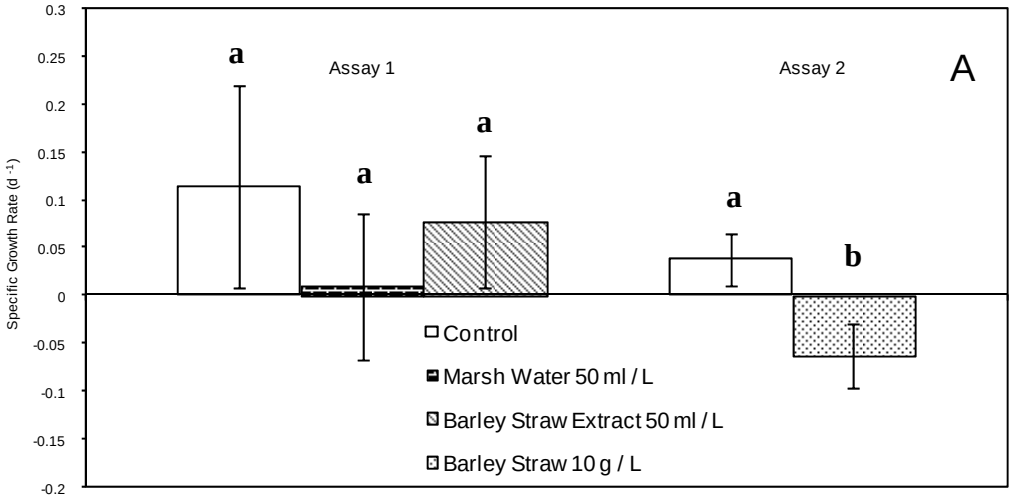


Figure 3. Comparison of *A. flos-aquae* growth rate for all mesocoms assays. Error bars show 95% confidence interval. Different letters represent significant difference ($\alpha = 0.05$) between treatments within each assay based on Tukey-Kramer multiple comparison test. Note, A) and B) have different scales.



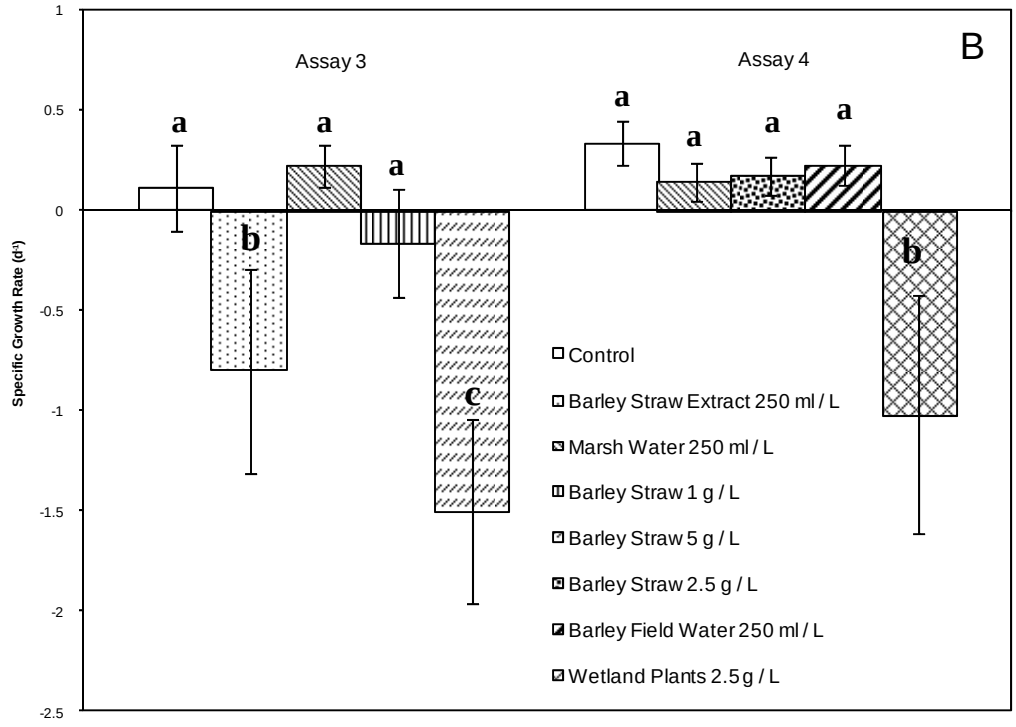


Figure 4. Average *A. flos-aquae* biovolume over time course of limnocorral assay. Error bars are SE. Control, Barely Straw n = 2; Klamath Marsh Water n = 3. Growth rates were calculated from linear regression of natural log transformed raw data. Control μ = 0.54 (± 0.43 95%CI); Barley Straw μ = -0.41 (± 0.43 95%CI); Klamath Marsh Water μ = 0.36 (± 0.57 95%CI).

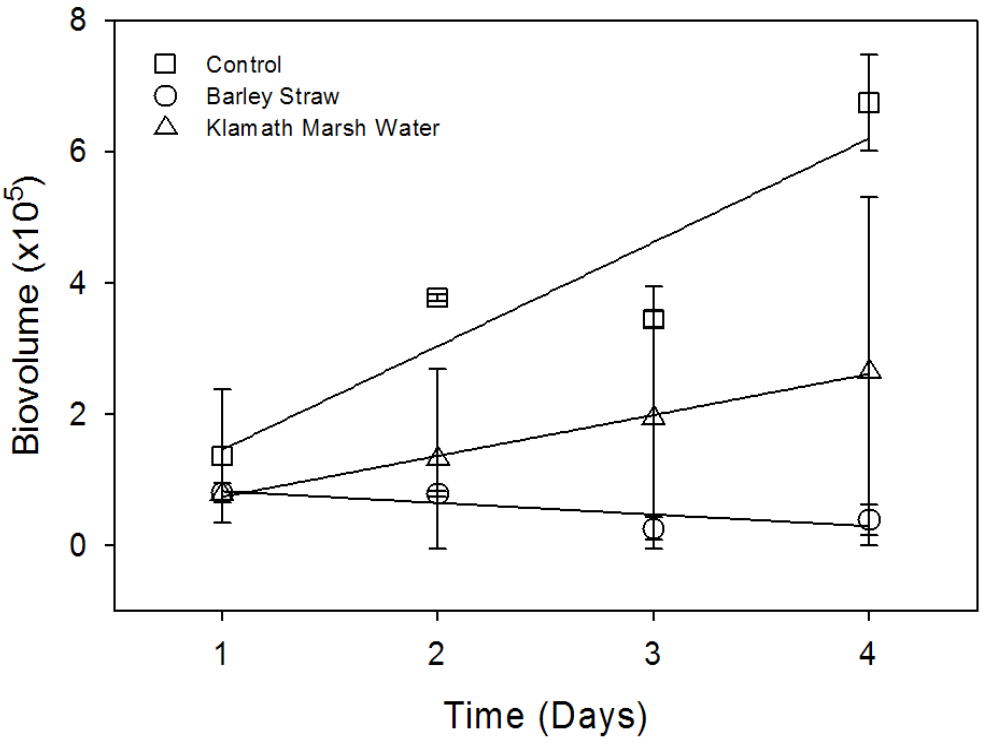


Figure 5. Response of *A. flos-aquae* average growth rate to varying concentrations of CDOM and UV exposure. Data from assays 3 and 4. High UV, Growth rate = $7.02 \times 10^{-4} + 0.42$; Low UV Growth rate = $2.41 \times 10^{-4} + 0.49$. High UV is 23 fold greater than low UV.

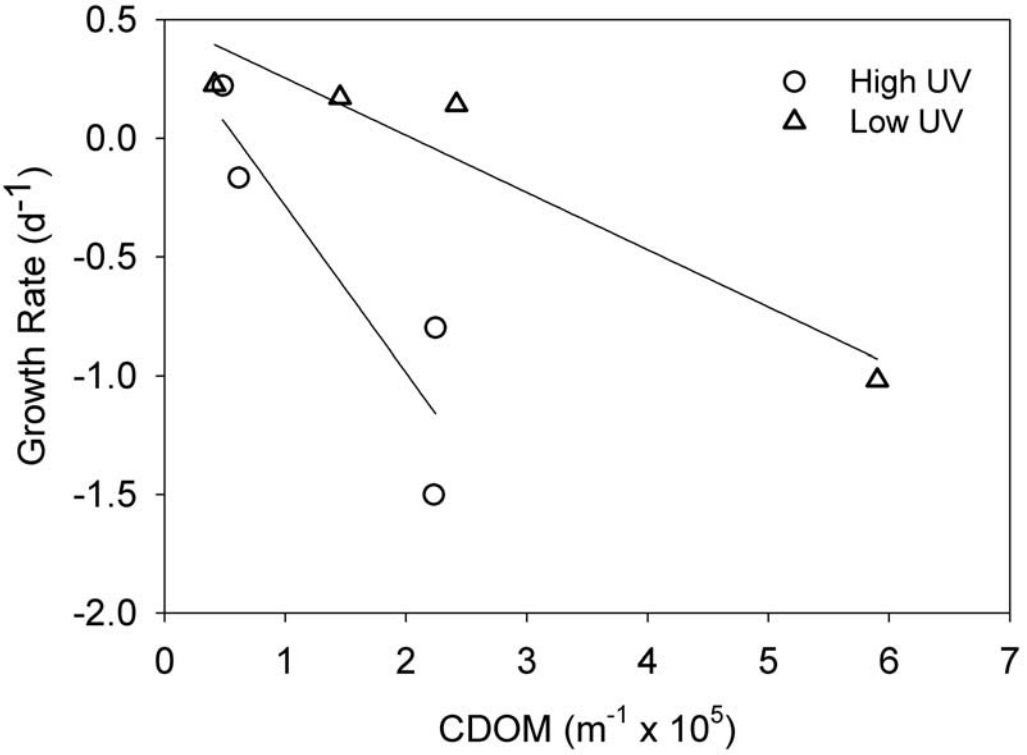


Table 1. Summary of experimental details for mesocosm assays 1 – 4 and limnocorral assay 1.

Experiment		Mesocosm 1	Mesocosm 2	Mesocosm 3	Mesocosm 4	Limnocorral 1
Date		Aug-05	Sep-05	Sep-06	Aug-07	Jul-06
Light	(µmol quanta m^{-2} s^{-1})	500	500	2000	500	2000
UV	(µW/cm2)	~1	~1	~23	~1	20-24
Temp	ºC	25D, 16N	25D, 16N	25	20	20-24
Number of replications		4	3	4	4	3
Barley Straw	source	-	Hyslop Farm	Klamath grown	Klamath grown	Klamath grown
	dose	-	10 g / L	5 g / L and 1 g / L	2.5 g / L	1.3 g / L
Barley extract	source	Hyslop Farm	-	Klamath grown	-	-
	dose	50 ml / L	-	250 ml / L	-	-
Barley field Water	source	-	-	-	Field adj. to UKL	-
	dose	-	-	-	250 ml / L	-
Marsh Water	source	Caledonia Marsh	-	Klamath Marsh	Klamath Marsh	Klamath Marsh
	dose	50 ml / L	-	250 ml / L	250 ml / L	80 ml / L
Dried Marsh Plant	source	-	-	-	Hanks Marsh	-
	dose	-	-	-	2.5 g / L	-

General Conclusions

These investigations have demonstrated that barley straw is effective at suppressing growth, or causing mortality, of the cyanobacterium *Aphanizomenon flos-aquae* in Upper Klamath Lake (UKL) and in controlled environments. These findings are based on replicated and repeated experiments, focusing on a single algal species. This represents a positive step beyond the many "before and after" observational and anecdotal reports circulating that barley straw is effective at controlling unspecified species of algae.

This work also shows that senesced wetland plant tissues suppress the growth and/or cause mortality of UKL *A. flos-aquae*. This finding supports the hypothesis that the loss of wetlands contributes to the massive annual blooms of *A. flos-aquae* in UKL. Not only are wetlands nutrient sinks, but have additional value in suppressing cyanobacterial blooms. These facts illustrate the need to conserve, maintain and restore wetlands.

Furthermore, this work demonstrates that there are two primary factors involved in the efficacy of suppression of *A. flos-aquae* by plant residues. These are the concentration of colored dissolved organic matter and light intensity. This provides evidence supporting the hypothesis that suppression of algal growth is due to cell damage from hydrogen peroxide and radical oxygen species produced by the photo degradation of colored dissolved organic matter.

This work also revealed that the addition of barley straw can have negative impacts on two water quality parameters. When added to a dense population of *A. flos-aquae,* low dissolved oxygen conditions can occur. Decrease in dissolved oxygen is due to bacterial decomposition of organic matter. However, at this time it is not known how much of the bacterial oxygen demand is for decomposing the dead AFA, and how much is for decomposing the barley straw. Barley straw additions also resulted in increased phosphate concentrations in treated water.

Cyanobacterial domination in UKL may be due to the loss of CDOM input, but the course of events in UKL is somewhat unique. Most freshwater bodies experiencing cyanobacterial harmful algal blooms (cyanoHABs) have not lost wetlands and have not shown marked changes in CDOM. In such cases, other factors are obviously responsible for the increase in freshwater cyanoHAB events. In many cases it appears that an increase of nutrients in water bodies is responsible. However there are examples of freshwater cyanoHAB events in high mountain and/or pristine lake systems with no identifiable sources of additional nutrients (e.g. agriculture or development). In these cases, the factors responsible cyanoHAB events are unknown and little work is being done to determine them.

The current response to most freshwater HAB events is reactionary. Only after a bloom is noticed are samples taken to identify the causative organism and, in some cases, to determine its density. In some instances further analyses are conducted to determine if toxins are present and in what concentration. Without a comprehensive record of water body characteristics such as temperature, pH, and water chemistry before and after HAB events, it will be exceedingly difficult, if

not impossible, to determine what factors are driving the occurrence of these events.

Due to the size of UKL it would be extremely difficult to treat the entire lake with barley straw. Targeted applications of barley straw or barley straw extract to areas where *A. flos-aquae* has accumulated would be feasible. These studies show that application of barley straw will reduce existing blooms of *A. flos- aquae*. However the ideal situation would be to suppress *A. flos- aquae* before it becomes so dense as to form a bloom. At this time we do not know what rate of barley straw would be effective in suppressing the onset of blooms, nor do we know how long such a barley straw treatment would remain effective.

A. flos- aquae is not the only HAB-causing cyanobacteria affecting lakes, ponds, reservoirs and rivers. Several other genera of toxigenic cyanobacteria are responsible for the 44 public health advisories the Oregon Department of Human Services has issued for freshwater bodies in the past five years. CyanoHABs are increasing in number and distribution. While the impact of these events has largely been on recreational activities, there have been cases where municipal water sources have been contaminated and rendered unusable. If the trend continues, more potable water supplies will be affected. As these other cyanobacteria continue to infest lakes and potable water reservoirs, methods of control with few or no adverse effects will need to be found. Considering its effect on *A. flos- aquae,* barley straw may also be effective on these other genera of cyanobacteria.

This series of experiments provides a general framework for assay of efficacy of barley straw on other HAB-causing organisms. However, important issues remain to be answered. These include (i) level of control achieved on different types of algae, (ii) assessment of prophylactic effects of straw applications, (iii) dose rates for prophylactic and symptomatic relief, (iv) degree of negative side effects (e.g. reduction in dissolved oxygen) as a function of dose, and (v) effects of light duration and quality.

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