

Potential for Direct Nitrate-Nitrite Inhibition of Submerged Aquatic Vegetation (SAV) in Florida Springs: A Review and Synthesis of Current Literature

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Abstract

Current observations of water quality in groundwater discharge from springs in Florida show anthropogenic enrichment of nitrate plus nitrite (NO_x-N) generally attributed to fertilizer application and/or wastewater or manure sources in individual spring sheds. Excessive levels of NO_x-N have been implicated in eutrophication of, and observed changes in, submerged aquatic vegetation (SAV) communities in several spring runs. While the indirect effects of nitrogen (N) enrichment on SAV, are well-documented (i.e., algal productivity resulting in shading of macrophytes), there is considerably less information available concerning direct effects of NO_x-N such as toxicity or inhibition of macrophyte growth. This manuscript constitutes a review of the pertinent literature and synthesis of the current understanding of elevated NO_x-N in aquatic systems and the effects on SAV as viewed from the prevailing eutrophication paradigm, as well as, explores the hypothesis that NO_x-N may have direct inhibitory effects on SAV growth in Florida springs.

Introduction

Phosphorus (P) is often considered the limiting nutrient in freshwater ecosystems,

and thus when available in excess, it is implicated as a causative agent in eutrophication (common eutrophication paradigm). Nitrogen (N), however, has been traditionally viewed by many as a lesser contributor to eutrophication of freshwaters, either because of the overshadowing nature of P issues or due to the ability of many cyanobacteria to fix atmospheric N, a process that significantly reduces perceived N limitation. This prevailing view stems from research conducted to elucidate the role of P in eutrophication (Schindler and Fee 1974; Schindler 1978) following the 1960s chemical industry claims of no effect of increased P in aquatic systems (Barker *et al.* 2008). More recently, several researchers have reasserted the view that N either alone or in concert with P, may exert ultimate control over algal productivity and subsequently macrophyte productivity in aquatic systems across the globe (Turpin 1991; Talling and Lemoalle 1998; Maberly *et al.* 2002; Clark and Baldwin 2002; James *et al.* 2003; James *et al.* 2005; Sagario *et al.* 2005; Dzialowski *et al.* 2005; Weyhenmeyer *et al.* 2007; Li *et al.* 2008). The extreme of this viewpoint suggests that N may have been the limiting nutrient in most northern hemisphere lakes and rivers prior to substantial N fertilizer utilization, which alleviated N limitation and catalyzed P limitation in enriched systems (Bergstrom

and Jansson 2006). A recent meta-analysis of published nutrient limitation studies found the number of N limitation cases to equal those of P limitation (Elser *et al.* 2007) and a significant number of cases of co-limitation. Moss (1990) contends that co-limitation was the normal condition prior to anthropogenic enrichment of nutrients. Recently, the role of N enrichment in alteration of ecosystem health has received renewed attention in aquatic ecosystems (Porter *et al.* 2013; Baron *et al.* 2013).

Numerous studies of eutrophication of freshwater ecosystems have observed a catastrophic shift from macrophyte to phytoplankton dominance after anthropogenic increases in available P (Wetzel 2001; Lacoul and Freedman 2005; Reddy and DeLaune 2008). The process involves rapid utilization of excess nutrients by phytoplankton and epiphytic algae, which results in explosive algal growth. The shift in primary productivity is also self-reinforcing, as turbidity increases with algal productivity, light becomes limiting to submerged aquatic vegetation (SAV) (Burkholder *et al.* 1992; Van den Berg *et al.* 1999). Death and decomposition of SAV only exacerbates excessive nutrient conditions by mineralization of organic nutrients bound in plant tissues.

Often, N and P have a positive synergistic effect on phytoplankton productivity. In a study by Sagarario *et al.* (2005), N and P additions alone did not show a significant effect, but when combined, the increase in phytoplankton and epiphytic algal biomass was dramatic. In addition to light attenuation by phytoplankton proliferation, epiphyte biomass burden and subsequent shading can be a primary causal mechanism for SAV mortality under eutrophic conditions (Borum 1985). In many spring runs in Florida, proliferation of epiphytic algae, as well as benthic macroalgae, have been observed concomitantly with declines of SAV communities (Stevenson *et al.*

2004; Frazer *et al.* 2006; Pinowska *et al.* 2007; Stevenson *et al.* 2007; Quinlan *et al.* 2008; Brown *et al.* 2008). Water quality in groundwater discharged from many springs in Florida has shown significant increases in $\text{NO}_x\text{-N}$ concentration, attributed predominantly to fertilizer application and/or wastewater or manure sources in individual spring sheds (Jones *et al.* 1996; Katz 2004; Albertin *et al.* 2012). Odum (1957) reported mean $\text{NO}_x\text{-N}$ concentration of 0.45 mg L^{-1} for Silver Springs in the 1950s which had risen to over 1 mg L^{-1} by 2005 (Munch *et al.* 2006; Quinlan *et al.* 2008). More dramatically, Rainbow River's $\text{NO}_x\text{-N}$ concentrations have increased from 0.08 to 1.22 mg L^{-1} (a 15 fold increase) over the last 50 years (Cowell and Dawes 2008). Interestingly, during this period of increasing $\text{NO}_x\text{-N}$, P concentrations have remained constant (Maddox *et al.* 1992; Scott *et al.* 2004) suggesting N to be the limiting nutrient in these aquatic systems. Perhaps more interesting is that not all springs with increasing $\text{NO}_x\text{-N}$ concentrations are experiencing increased algal productivity (Heffernan *et al.* 2010), but many are experiencing declines in SAV health. These observations, while somewhat inconsistent with the common eutrophication paradigm, have prompted several hypotheses as to the role of increased nitrogen availability in the observed loss of SAV in spring systems statewide.

This manuscript reviews these competing hypotheses concerning the relationship between elevated $\text{NO}_x\text{-N}$ and observed ecological changes, specifically declines in SAV coverage and increases in epiphytic and benthic algae, in regional spring ecosystems. Significant attention is given to potential inhibitory effects of elevated $\text{NO}_x\text{-N}$ on SAV growth in springs and possible mechanisms for this inhibition are discussed. These mechanisms focus on the assimilative nitrate reduction process, resulting buildup of toxic ammonia (NH_3), and energetic consequences of unregulated

NO_x-N uptake on SAV. This discussion concludes with a call for research to clarify the role of elevated NO_x-N in the observed degradation of SAV communities.

Competing Hypotheses

The initial hypothesis (H₁) posited by members of the scientific community, as well as the general public, was that the increase in N availability, observed as NO_x-N concentration in spring waters, alleviated N limitation and therefore was responsible for a shift in primary productivity from SAV to epiphytic algae and benthic macroalgae. While there have been studies to report N utilization by algal mats in Florida springs (Cowell and Botts 1994; Cowell and Dawes 2004; Albertin 2009; Sickman *et al.* 2009), there have been several observations that contradict the common eutrophication paradigm, namely the lack of significant increase in other forms of N (Cohen *et al.* 2007) or P (Maddox *et al.* 1992; Scott *et al.* 2004) in spring waters. Brown *et al.* (2008) concluded that there was insufficient evidence to link nitrate enrichment to changes in algal cover. Heffernan *et al.* (2010) argue convincingly that studies by Canfield and Hoyer (1988) and Duarte and Canfield (1990) found no relationships between nutrients and total vegetative biomass in spring runs as would be expected under nutrient limitation scenarios, and that recent surveys of algal biomass have not found any significant linkage of biomass to N or P concentrations (Stevenson *et al.* 2004; Stevenson *et al.* 2007). Further, Heffernan *et al.* (2010) and Liebowitz (2013) report stronger relationships between dissolved oxygen (DO), grazer populations, and algal abundance than with nutrients.

Heffernan *et al.* (2010) documents a decline in water column DO in Silver Spring and suggests looking to other drivers of algal proliferation in spring systems, including DO control of invertebrate grazers that may result in altered trophic structure in springs to favor algal dominance. Liebowitz (2013)

reports a significant negative association between algal and gastropod biomass in Florida springs suggesting top down control of algae by invertebrate grazers, a finding supported by several studies of grazer control of algae in other systems (Hildebrand 2002; Heck and Valentine 2007; Gruner *et al.* 2008; Baum and Worm 2009; Estes *et al.* 2011). Further, Liebowitz (2013) also found a significant relationship between dissolved oxygen (DO) and gastropod biomass in a survey of 11 springs, suggesting DO has a significant indirect effect on algal biomass via controlling grazer abundance and/or activity. Under low flow or current velocity conditions, nutrient enrichment and subsequent algal growth may outpace grazer pressure resulting in severe light reductions (Harlin and Thorne-Miller 1981). Alternatively, under similar nutrient enrichment and moderate to high flushing or exchange of water (as in lotic or tidally influenced systems), herbivores have been observed to control epiphytic algal biomass (Neckles *et al.* 1993). Liebowitz (2013) argues that hysteretic responses of grazer populations to disturbances could be responsible for the over abundance of algae in springs where no clear grazer stress is present. For instance, invasive plant control measures utilizing herbicides and copper compounds are widely employed with known negative impacts on grazer populations (Evans 2008). Such a disturbance could enable algal populations to exceed thresholds for grazer control. This gives rise to a second hypothesis (H₂) that grazer control of algae in springs has been altered by DO in some cases and by episodic or unknown exposures to other stressors.

The presence of herbicides or other agrochemicals that may be inhibitory to either algal grazers or macrophytes themselves supports a third hypothesis (H₃) which states that a “nitrate cohort” defined as substance[s] associated with the same mechanisms involved in increased nitrate concentration, such as changes to land

use (concentrated feed lots for cattle) or ineffective waste treatment (spray fields or failed septic tanks) has an inhibitory or toxic effect on SAV. The widespread use of agrochemicals such as commercial pesticides, fungicides and herbicides increases potential for these compounds to impact spring ecosystems. Several anthropogenic organic compounds have been detected in springs; however, the low levels observed did not elicit alarm (Phelps *et al.* 2006; Phelps 2004). Recent increases in consumer use of compounds such as atrazine, a herbicidal fertilizer additive, (Ackerman 2007) and triclosan, an antimicrobial agent, (Fulton *et al.* 2010) suggest these compounds may have deleterious effects on SAV (and/or grazer population dynamics).

A fourth and least well understood hypothesis (H_4) is that nitrate itself has an inhibitory (direct) effect on SAV growth resulting in a shift to algae dominated system. The accepted view of nitrate and ammonia combined with P to create conditions for algal dominance and subsequent shading out of macrophytes (Mulligan *et al.* 1976) is that of an indirect effect. However, a direct effect of nutrient enrichment has been suggested as a factor in macrophyte disappearance in aquatic systems undergoing enrichment (Genevieve *et al.* 1997; Farnsworth and Baker 2000). Several authors (Klotzli 1971; Schroder 1979; Boar *et al.* 1989), have reported correlation of reed bed disintegration and increases in nitrate loading to lakes in England. Decreases in *Phragmites australis* root and rhizome production was observed in concert with increased nitrate loading (Ulrich and Burton 1985). Ulrich and Burton (1985) also reported that nitrate stimulated growth and overall biomass increased with increased nitrate availability, however, below ground biomass production (roots and rhizomes) did not increase at concentrations up to 6 mg $\text{NO}_x\text{-N L}^{-1}$. These $\text{NO}_x\text{-N}$ concentrations resulted in significant decreases in below ground to above ground biomass ratios and

resulted in an overall decline in health of the reed stands. Nitrate to potassium ratios in surface waters and in tissues are correlated with highest degree of degradation of *Phragmites australis* beds (Boar *et al.* 1989), however, the causative mechanism is unknown as is the potential for synergistic effects of increased availability of N and K. Because the aforementioned species is an emergent macrophyte, free of algal shading, it serves as a significant indicator of potential inhibitory effects of $\text{NO}_x\text{-N}$ on plant growth. An in depth review of current literature suggests several authors have observed apparent direct inhibitory effects of $\text{NO}_x\text{-N}$ on SAV in both marine and freshwater environments. The remainder of this paper will synthesize the available literature and investigate potential causal mechanisms for the observed negative effects of elevated $\text{NO}_x\text{-N}$ on SAV.

Evidence of Direct Effects of Nitrate on SAV

Opportunistic luxury consumption of nutrients is characteristic of SAV and thus accumulation in tissues is anticipated for macrophytes adapted to limited nutrient availability, such as seagrasses and some freshwater SAV (Wetzel 2001). The prevailing viewpoint is that most macrophytes acquire nitrogen via roots (Cedergreen and Madsen 2003), however, foliar absorption is also a viable mechanism when sediment sources are not available or abundant (Barko and Smart 1986). When ammonium nitrogen ($\text{NH}_4\text{-N}$) concentration exceeds 0.1 mg L^{-1} , macrophytes preferentially use $\text{NH}_4\text{-N}$ (Nichols and Keeny 1976). Hence, the dominant form of N utilized by most SAV is $\text{NH}_4\text{-N}$. However under N limitation nitrate is also utilized, predominately from the water column. Due to its abundance, $\text{NO}_x\text{-N}$ is the presumed dominant form for N uptake by SAV in Florida springs. Several researchers have made qualitative

observations of SAV inhibition closest to spring vents where $\text{NO}_x\text{-N}$ concentrations are highest (Munch *et al.* 2006; Mattson pers. observation). Similarly, several authors suggest observed declines in macrophytes in other systems was a direct effect of increased $\text{NO}_x\text{-N}$ (Burkholder *et al.* 1992; Burkholder *et al.* 1994; Wang *et al.* 2012), suggesting closer investigation of this phenomenon is warranted.

Burkholder *et al.* (1992) report that *Zostera marina* (eelgrass) exhibited highly negative physiological effects (even death) when dosed with 0.05, 0.1 and 0.5 mg L^{-1} $\text{NO}_x\text{-N}$. Although a marine species, this plant shows extreme sensitivity to increased nitrate evidenced through loss of carbon storage in roots unrelated to shading by algae. The apparent lack of an inhibition or regulation mechanism of nitrate uptake by eelgrass (Roth and Pregnall 1988) was implicated in the observed disruption of internal nutrient ratios, presumably due to carbon expenditure in amino acid synthesis to reduce intracellular ammonia toxicity. Hierarchical partitioning analysis of water quality parameters found $\text{NO}_x\text{-N}$ exerted the greatest detrimental effect on charophyte occurrence in wetlands of the UK (Lambert and Davy 2011). *In situ* studies of *Chara globularis* showed that it was extremely sensitive to nitrate with maximal relative growth rate observed at 0.5 $\text{mg NO}_x\text{-N L}^{-1}$ and a linear decline in growth with higher concentrations. At 6 $\text{mg NO}_x\text{-N L}^{-1}$, growth was severely limited, similar to results of no $\text{NO}_x\text{-N}$ treatment (Lambert and Davy 2011). Similarly, biomass accumulation was strongly inhibited by nutrient accumulation (N) in *Potamogeton maackianus* A. Been (Ni 2001). The most definitive observations of inhibition were in the form of shrinkage of parenchyma tissues and disappearance of starches and chloroplasts observed in increased $\text{NO}_x\text{-N}$ and $\text{NH}_4\text{-N}$ concentration treatments of *Vallisneria natans* (Wang *et al.* 2012.). It is important to note that ammonia, whether sourced from high

concentrations in water or derived (via intracellular nitrate reduction) from elevated nitrate availability to SAV is the source of toxicity.

The paradigm of nitrogen effects on water clarity often overshadows potential direct effects of excess N on SAV. For instance, Sagrario *et al.* (2005) reported that high N is not directly inhibitory to *Potamogeton pectinatus* L., *Elodea canadensis* and *Nymphaea sp.* at 10 mg L^{-1} of total N (TN) due to overpowering effects of increased algal shading. However, closer inspection of the results indicates moderate dosing of 4 mg TN L^{-1} resulted in decreased growth with respect to controls under equal or better water clarity, a noteworthy result that went unmentioned. Further, summer TN levels declined significantly in mesocosms truncating the duration of exposure for macrophytes, which likely confound interpretation of the results by the authors. In a study by Li *et al.* (2008), $\text{NO}_x\text{-N}$ additions were noted to increase *Vallisneria spinulosa* biomass over control at 2.5, 5.0, 7.5 mg L^{-1} concentrations in water column but at 10 mg L^{-1} growth was not significantly different from control (1 mg L^{-1}) suggesting some inhibition of growth. It is unclear why the authors did not conclude that a $\text{NO}_x\text{-N}$ threshold had been exceeded between 7.5 and 10 mg L^{-1} . This lack of interpretation by some authors is likely due to a strong focus on algal production and subsequent shading, not direct effects of nitrate on SAV (Sturgis and Murray 1997). Further, variability among species with respect to effects of $\text{NO}_x\text{-N}$ appears to be high (Burkholder *et al.* 1994). This is exemplified by conflicting reports on potential inhibition of macrophyte growth by excessive water column nitrate (Li *et al.* 2005). Best (1980) reported no inhibition of *Ceratophyllum demersum* at concentrations of up to 105 $\text{mg NO}_x\text{-N L}^{-1}$ but did observe ammonia toxicity at 45 $\text{mg NH}_4\text{-N L}^{-1}$. This finding suggests that *C. demersum* is well suited to luxury uptake

of N. Conversely, Lambert and Davy (2011) assert a mean annual concentration limit of 2 mg NO_x-N L⁻¹ is necessary to protect charophytes.

Our review of available literature did not find studies of N enrichment with SAV species common in Florida spring systems (*Vallisneria americana*, *Sagittaria kurziana*, *Najas* spp., *Potamogeton* spp.). However, the potential mechanism of inhibition, which likely varies among species, are discussed here in general terms for SAV and are viewed as potential mechanisms until tested on individual species of interest.

Potential Mechanisms of Inhibition

Nitrate toxicity has been well documented for vertebrate animals (including humans) (Kim-Shapiro *et al.* 2005) as well as invertebrates (Mattson *et al.* 2007). However, the potential of NO_x-N toxicity or inhibition of SAV is not well understood, nor is it intuitive given our understanding of mechanisms of toxicity for higher organisms. Observations coinciding with elevated inorganic N (NO_x-N and or NH₄-N) in plants include stunted growth, iron deficiency, amino acid accumulation, oxidative stress and structural tissue damage (Burkholder *et al.* 1992; Smolders *et al.* 1997; Smolders *et al.* 2000; van der Heide *et al.* 2008; Wang *et al.* 2012). To better determine potential inhibitory mechanisms of NO_x-N, a closer look at the process of nitrogen assimilation is necessary.

Assimilatory Nitrate Reduction

Most aquatic plants absorb nitrate, which is then sequentially converted to nitrite and then ammonium by the nitrate reductase system (Salisbury and Ross 1992). In SAV, before nitrate can be utilized by the plants, it must be converted to ammonium by a series of sequential enzyme mediated reactions

(Figure 1) involving nitrate reductase and nitrite reductase (Guerrero *et al.* 1981). This process is termed assimilatory nitrate reduction (ANR) and results in ammonia being incorporated into amino acids. Genetic or environmental factors, such as light, temperature, depth, pH, and location within vegetated patch (edge versus center) (Roth and Pregnall 1988; van der Heide *et al.* 2008), can modulate this series of biochemical reactions resulting in a high level of variability among species with respect to nitrate reduction processes and rates (Pate 1980; Guerrero *et al.* 1981). Water temperature can be problematic for SAV by increasing respiration rates and impairing enzyme function (Zimmerman *et al.* 1989; Lacoul and Freedman 2006; Riis *et al.* 2012), thus impairing nitrogen assimilation. However, SAV in spring runs generally do not experience thermal stress due to the thermal consistency of groundwater (unless exposed in shallow backwaters).

Uptake of NO_x-N is driven primarily by external nitrate concentrations (Marschner 1998) and in aquatic macrophytes, increased water column concentrations of NO_x-N results in significant increases in nitrate reductase activity (NRA) (Cedergreen and Madsen 2003). Studies of *Zostera marina* indicate newer leaves are more active with respect to NRA and rates between individual plants can be variable with a 2-3 fold difference (Roth and Pregnall 1988). It has been suggested that differences between root and shoot NRA depends upon uptake rates of individual species (Gojon *et al.* 1994) and that location of nitrate reduction (root or shoot) is also species specific (Cedergreen and Madsen 2003). From an energetic standpoint, photosynthetic tissues would be a more advantageous location for NRA to occur due to a lack of need to transport NO_x-N to the roots (Raven 1985; Schjoerring *et al.* 2002) and this appears to be the case for SAV (Roth and Pregnall 1988).

Once uptake has occurred, there are physiological deterrents to storing nitrate freely within the cytoplasm due to osmotic stress (Salisbury and Ross 1992). Therefore nitrate is stored in vacuoles or rapid conversion to ammonia occurs within the cytoplasm (Heimer and Filner 1971; Wagner 1979; Granstedt and Huffaker 1981). These storage vacuoles are not, however, without their limitations. Hydration of vacuolar materials such as nitrate or associated cations (K, Na) can require significant water, which ultimately limits the concentration that can be stored intracellularly (Raven and Smith 1976). Regardless of the storage method of nitrate, the reduction of nitrate and subsequent production of ammonia requires plants to avoid toxicity by allocating carbon and energy to protein (amino acid) synthesis to alleviate ammonia buildup (Salisbury and Ross 1992). Under normal exposure to NO_x-N, ANR uses approximately 25% of the reductant energy

produced by photosynthesis and root/shoot respiration (Crawford 1995).

Closer inspection of the biochemical pathways for ANR reveals some significant differences between SAV and filamentous macroalgae, the two competing primary producers in many springs. Assimilatory nitrate reductase activity in green algae and higher plants is dependant upon NAD(P)H for reducing power (Figure 2A). Further, the negative feedback inhibitor of the nitrate reductase enzyme in some species is nitrite, which competitively binds with nitrate reductase. This is not the case for all species as reported by Roth and Pregnell (1988) who documented the inability of *Zostera marina* to “turn off” or regulate nitrate reductase, a very critical observation with respect to the potential for some SAV to moderate this enzyme as it establishes evidence that some species may not have the capacity to regulate NRA. Cyanobacteria,

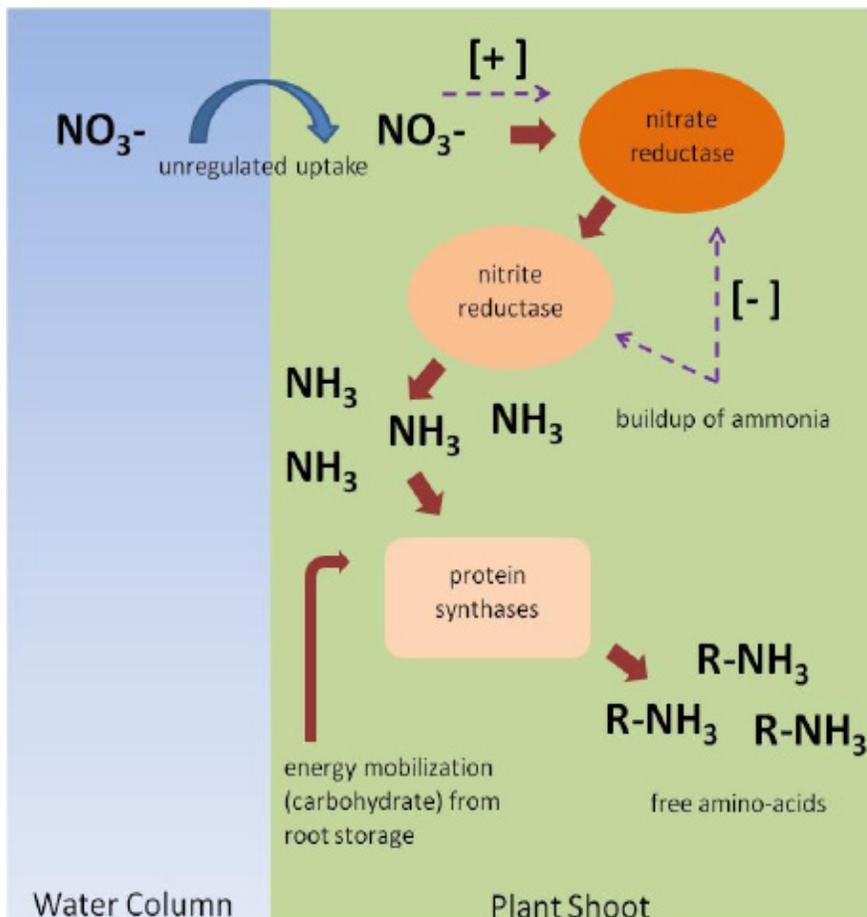


Figure 1. Conceptual model of nitrate overload hypothesis. Uptake of nitrate is unregulated at the cellular level and presence of nitrate induces nitrate reduction to ammonia. Buildup of ammonia should be a negative feedback[-] for nitrate reduction enzymes; however this process appears not to function in some species. Ammonia can be toxic to plants and therefore is alleviated via amino acid and consequent protein synthesis, which requires energetic inputs from plant carbohydrate stores. Buildup of free amino acids and depletion of root carbohydrate stores are potential diagnostics of nitrate overload in SAV.

on the other hand, cannot utilize reduced pyridine nucleotides as do green algae and higher plants. The alternative electron donor for algal nitrate reductase (Figure 2B) is ferredoxin (Guerrero *et al.* 1981). This reaction appears to give cyanobacteria a slight energetic advantage as the ΔG of the reaction is 4.6 Kcal greater per mole for ferredoxin mediated reduction versus NAD(P)H. The second reduction reaction, nitrite reduction to ammonium, is very similar in all photosynthetic organisms and utilizes ferredoxin as the electron donor specifically.

Ferredoxin requires iron in its structural complex, thus increased iron concentration

in springs may also provide cyanobacteria a competitive advantage over green algae and SAV. This is due to the inability of the latter organisms to utilize ferredoxin in nitrate reduction. Smolders *et al.* (1997) report iron deficiency in SAV exposed to higher levels of $\text{NO}_x\text{-N}$, presumably due to the need for ferredoxin in nitrite reduction. Because NAD(P)H also serves as reducing power for many other metabolic reactions, utilization of NAD(P)H for nitrate reductase results in a decrease of its use in other metabolic reactions and potential buildup of other metabolites within cells, which may reduce plant growth and metabolic processes (Lea and Mifflin 1979). Of greater

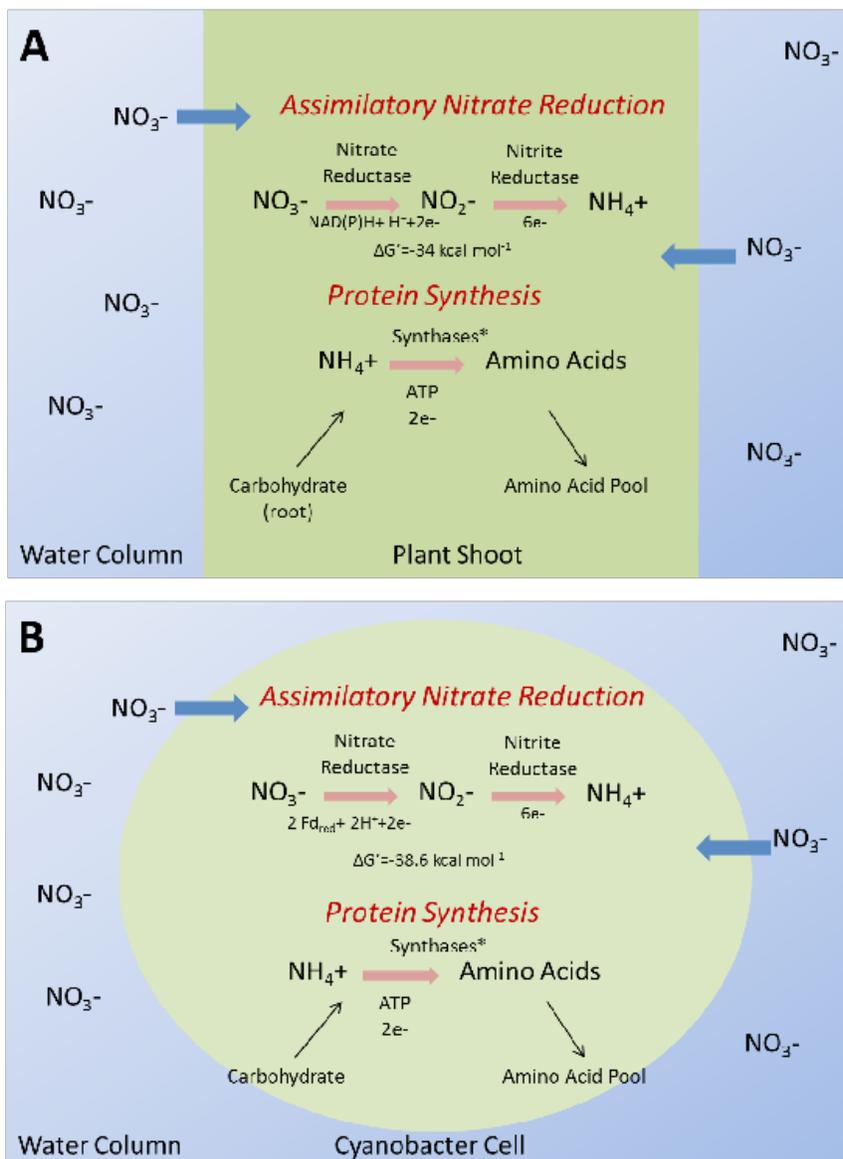


Figure 2. Assimilatory nitrate reduction in [A] SAV shoot and [B] cyanobacterial cell. Note cyanobacteria cannot utilize NAD(P)H as an electron donor in the reduction of nitrate to nitrite and thus rely solely upon ferredoxin. Also note a slight energetic advantage [$\Delta G'$] exists for cyanobacteria in the reduction of nitrate to nitrite.

concern, the accumulation of ammonia, the end product of ANR, can be extremely detrimental to photosynthetic organisms. Excessive intercellular ammonia represents a significant source of toxicity for SAV through inhibition of respiration, metabolic activities, and compromising of cell membrane integrity. This toxicity may be realized when excess nitrate availability is combined with the possibility of poorly regulated ANR, resulting in ammonia buildup in tissues. ()

Ammonia Toxicity

Ammonia toxicity is well documented in terrestrial plants (Salisbury and Ross 1992) as well as in seagrasses and other hydrophytic vegetation (Katwijik *et al.* 1997; Hemminga and Duarte 2000). In most plants, excessive ammonia is toxic due to inhibition of respiration, photosynthesis and other metabolic processes. Ammonia toxicity is often indicated by a decrease in soluble sugars in the cytoplasm (Cramer and Lewis 1993) or excessive tissue concentrations of ammonium that exceed the plants' ability to incorporate into amino acids (Meher and Mohr 1989). Inhibitory effects of high ammonium on SAV have been documented (Best 1980; Smolders *et al.* 1996) and implicated in succession of freshwater SAV communities (Schuurke *et al.* 1986; Brouwer *et al.* 1997; Clarke and Baldwin 2002). Excessive ammonium can inhibit photosynthesis (Cao *et al.* 2004) resulting in diminished photosynthate (soluble sugar) production and lead to necrosis in some macrophytes (Smolders *et al.* 1996). To alleviate $\text{NH}_4\text{-N}$ stress, plants must convert the free ammonium to amino acids via biosynthesis (Figures 1 and 2). This process has an energetic cost, requiring carbon and energy inputs from the plant (i.e. soluble sugars, photosynthate). Lambert and Davy (2011) invoke energetic expenditure in regulating ammonia internally as a likely cause of growth decline in *Chara*

sp. exposed to $\text{NO}_x\text{-N}$ in excess of 2 mg L^{-1} . Water column ammonia concentrations $>1 \text{ mg l}^{-1}$ resulted in decreased soluble sugar content in *Potamogeton crispus* and increased soluble amino acids (Cao *et al.* 2004). Interestingly, in a study by Cao *et al.* (2004), responses of amino acids and soluble sugar indicators of ammonia stress were dependant upon duration of exposure. Further, activity of ascorbate peroxidase and superoxide dismutase (both anti-oxidant enzymes) were highest at 1 mg L^{-1} ammonium and decreased significantly as ammonium increased (Cao *et al.* 2004). Other factors are known to influence the degree of ammonia toxicity in plant tissues. For instance, Netten *et al.* (2013) reported that light and temperature also had significant effects on toxicity of ammonia in *Elodea Canadensis*. While temperature and water column ammonia concentration are not issues for the spring ecosystems discussed here (Munch *et al.* 2006), the availability of $\text{NO}_x\text{-N}$ can provide excessive nitrogen to SAV.

The energetic demand of reducing the resulting ammonia toxicity, in concert with unregulated ANR, could represent a very significant stress on SAV (Smolders *et al.* 2000; Wang *et al.* 2012).

Amino-Acid Synthesis

Ammonia is incorporated into α -amino acids by way of one or both known pathways (Figure 2), the glutamate dehydrogenase and the glutamate synthetase-glutamate synthase pathway (Guerrero *et al.* 1981). Buildup of free amino acids in tissues is considered an indication of "nitrogen overload" or impending toxicity due to excessive nitrogen availability (Smolders *et al.* 1996; Smolders *et al.* 2000; Wang *et al.* 2012). Specific types of amino acids that accumulate in tissues (for instance arginine, glutamine, asparagines) are dependant on the stresses involved (toxicity, mineral deficiency, grazer pressure) and the species

of SAV (Rabe and Lovatt 1986; Rabe 1990; Marschner 1998; Smolders *et al.* 2000). Significant evidence of the nitrogen overload hypothesis is presented by Wang *et al.* (2012) who reported reduction of aerenchyma tissue, chloroplasts and starch grains in tissues of *Vallisneria natans* exposed to increased nitrate and ammonia levels. The authors contend that loss of structures and starch content is related to photosynthate required to reduce nitrate to ammonia and further sequester toxic ammonia in amino acids, a process that requires significant energy expenditure by plants. Due to the high energetic demand, $\text{NO}_x\text{-N}$ overload may perhaps lead to susceptibility to pathogens. For example, *Zostera marina*, as well as some other angiosperms, are known to decrease production of antimicrobial compounds such as phenolics during times of increased protein synthesis associated with N enrichment (Buchsbaum *et al.* 1990).

Summary of Mechanisms of Inhibition

Review of the current literature concerning $\text{NO}_x\text{-N}$ effects on SAV is compelling in that the process of ANR is highly variable among species and the potential for unregulated uptake, an adaptation ostensibly stemming from luxury uptake, could induce the “nitrogen overload” condition (Smolders *et al.* 1996; Smolders *et al.* 2000; Boedeltje *et al.* 2005; Wang *et al.* 2012). The resulting accumulation of ammonia, the end product of ANR, can itself be a significant stressor to plants or, by necessitating protein synthesis to alleviate ammonia stress, can cause depletion of SAV carbohydrate stores (Guerrero *et al.* 1981; Wang *et al.* 2012). In other aquatic ecosystems (ponds, lakes) that are finite in the overall mass of N, potential $\text{NO}_x\text{-N}$ toxicity may be reduced based upon density of SAV (van der Heide *et al.* 2010). However, under the unique lotic conditions of springs (increasing nitrate concentrations

and constant exposure due to high flow conditions), the potential for increased biomass of SAV to ameliorate potential toxicity is not anticipated. Determining these direct effects of $\text{NO}_x\text{-N}$ on SAV native to Florida springs will be of primary importance to directing management effort with respect to springs restoration.

Ecological Implications

Globally, many aquatic ecosystems have been altered, some seemingly irrevocably, by the anthropogenic addition of excessive nutrients (N and P). For example, in both temperate and tropical lakes undergoing nutrient enrichment, catastrophic shifts from macrophyte to phytoplankton dominance have been observed with regularity in the last half century. In Florida, significant effort has been invested in ameliorating these catastrophic shifts on large lakes such as Apopka (Dunne *et al.* 2012) or Okeechobee (James *et al.* 2011; Harwell and Sharfstein 2009). This shift in primary productivity has resounding effects throughout the food web. Further, habitat loss and susceptibility to altered environmental conditions (for example: hypoxia, shifts in pH) can have detrimental effects on established flora and fauna. This shift in primary productivity may also impart significant changes to ecosystem services such as biogeochemical cycling of nutrients and habitat quality for fauna. Similarly, Florida’s springs systems, which have immense ecological, cultural and economic value to the state have undergone significant ecological degradation in recent decades. Therefore, concern exists for determining the relationship between these changes and the observed increase in $\text{NO}_x\text{-N}$ in springs. Of primary concern is elucidating the role nitrate enrichment has had (whether direct or indirect via synergistic interactions with other stressors) in the observed decline of these systems.

Research Needs

Research is warranted to investigate if SAV native to Florida springs are experiencing any inhibitory effects due to elevated $\text{NO}_x\text{-N}$ concentrations by one or more of the proposed mechanisms: 1) unregulated $\text{NO}_x\text{-N}$ uptake and reduction, 2) ammonia toxicity from excess accumulation in vivo, and 3) carbohydrate depletion from intercellular or root storages. Of primary interest would be a synoptic sampling of SAV from several springs along gradients of elevated $\text{NO}_x\text{-N}$, as well as sampling springs without significant increases of $\text{NO}_x\text{-N}$, to determine if gradients of inhibition or energetic stress is observed. Species of interest are *Vallisneria americana*, *Sagittaria kurziana*, *Najas sp.*, *Potamogeton sp.*, *Ceratophyllum spp.*, *Hydrilla sp* and *Chara spp*. Biometrics such as root and shoot biomass and calculation of root:shoot mass ratios may be the most informative preliminary measurement approach. If these preliminary results suggest significant impact to growth from elevated $\text{NO}_x\text{-N}$, then more intensive studies may be indicated. These studies might include, but are not limited to, determination of direct use of $\text{NO}_x\text{-N}$ by SAV, toxicity thresholds for $\text{NO}_x\text{-N}$ (via $\text{NH}_4\text{-N}$) in tissues, and potential synergistic effects of $\text{NO}_x\text{-N}$, K, and Fe. With consideration of the significant changes occurring in Florida Spring ecosystems, other potential contributors to algal proliferation (indirect effect on SAV health) such as declining DO and multiple stressor hypotheses also warrant investigation.

Discussion with Reviewers

1. Reviewer 2: *Could the authors add a comment on the molecular basis for ammonia toxicity in animals (Monfort et al. 2002)?*

While this contribution deals specifically

with submerged aquatic vegetation, the authors realize that internal physiological nitrate/nitrite ($\text{NO}_x\text{-N}$) reduction to ammonia by invertebrates in the same ecosystem could bring about a loss of top down control of algae (and concomitant decline in SAV) if algal grazing organisms were negatively affected by increased $\text{NO}_x\text{-N}$. Monfort et al. (2002) present a molecular basis for ammonia toxicity in which activation of N-methyl-D-aspartate (NMDA) is indicated during excessive ammonia exposure. Strong evidence is presented supporting the assertions that NMDA receptors are responsible for the following effects (from Monfort et al. 2002): exhaustion of ATP levels in the brain; dephosphorylation and activation of $\text{Na}^+/\text{K}^+\text{-ATPase}$ in brain; impairment of mitochondrial function and calcium homeostasis which decreases ATP synthesis; reduction of glutamine synthetase activity which reduces ammonia elimination in the brain. Combined these effects reduce brain activity and neuronal degeneration and eventual mortality. The process outlined by Monfort et al. (2002) has application to this work in that it could be an additional stressor on the grazer community resulting in unchecked algal growth (a common observation in springs with impaired SAV). Moreover, the process described alters ATP availability and production in the brain tissue of invertebrates and is similar in that respect to the process proposed in our manuscript which invokes the depletion of photosynthate stores in SAV tissues during amino acid synthesis which is required to reduce intracellular levels of ammonia resulting from unchecked $\text{NO}_x\text{-N}$ reduction.

Monfort P, Kosenko E, Erceg S, Canales J-J, Felipe V (2002) Molecular mechanism of acute ammonia toxicity: role of NMDA receptors. *Neurochemistry International* 41(2-3): 95-102

2. Reviewer 2: *The authors also ought to consider a molecular basis for ammonia*

toxicity, resulting from the Maillard and the Strecker reactions.

The authors did not describe the molecular basis for ammonia toxicity in the paper, rather for brevity sake, simply referred to its occurrence only. The actual mode of toxicity from accumulating ammonia in the cells of SAV may be sourced from intracellular production of physiologically disrupting compounds produced by common sequential reactions of amino acids sourced from large quantities of free ammonia building up in plant cells. Hence, Maillard and Strecker reactions have relevance to this discussion. First, the Maillard reaction is a chemical reaction that produces glycosamine from amino groups of amino acids reacting with carbonyl groups of reducing sugars within the cell. Glycosamine is an unstable product and thus undergoes further reactions (Amadori series rearrangement) which yields ketosamines. These compounds, after undergoing further dehydration and de-amination, produce di-carbonyls. The Strecker reaction is involved when the di-carbonyl products react further with readily available amines to produce acrylamide, a known carcinogen and cellular disruptor. This is especially the case when asparagine is present (a dominant amino acid observed in SAV under high nitrate conditions by Wang et al. 2012). Thus, while the authors observe increased nitrate/ nitrite in the spring ecosystems and focus on the role this NO_x-N may have on SAV in the systems, the actual process is a bit more complicated in that NO_x-N reduction to ammonia is not in itself phyto-toxic, rather, the buildup of ammonia and resulting reactions such as the Maillard and Strecker reactions, can produce toxicity at the cellular level. We hypothesize here that SAV, in an effort to alleviate toxicity of lesser regulated ammonia production, utilizes stored energy from photosynthate to produce amino-acids. The authors recognize that under specific conditions, either mechanism could produce the observed stunting (and possibly

loss) of SAV observed in Florida Springs.

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