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Effects of Nutrient Pre-Exposure on Atrazine Toxicity to *Vallisneria americana* Michx. (Wild Celery)

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Abstract Accelerated eutrophication is common to many freshwater and marine environments and often co-occurs with the presence of anthropogenic chemicals. However, the toxic effects of common chemical stressors such as herbicides in the presence of elevated nutrients are not well understood for most aquatic flora, particularly vascular species. To provide insight, field-collected *Vallisneria americana* Michx. (wild celery) were sequentially exposed to three nutrient concentrations for 3 months and then to nominal 11 and 110 μg L^{-1} atrazine for 96 h. Nutrient concentrations (combined NH_4^+, NO_2^-, NO_3^-, PO_4^-) were based on ambient concentrations in the St. Johns River (FL) and ranged from 0.013 to 0.668 mg L^{-1}. Nutrient pretreatment potentiated the toxicity of atrazine as determined by chlorophyll fluorescence activity. Electron transport rates (ETR) were significantly less (48–59%) for plants pretreated with low and ambient nutrient levels in the presence of an average of 107.5–128.1 μg L^{-1} atrazine. Significant ETR reductions were also observed for plants exposed to an average of 11.4 μg L^{-1} atrazine after exposure to nutrients three times the ambient concentration in the St. Johns River. The results indicate the importance of considering the presence of nutrients in chemical hazard assessments, particularly for phytotoxicants and nontarget vascular plants.

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Aquatic plant-dominated ecosystems are of increasing interest to the scientific community due to their ecological and economic value (Millennium Ecosystem Assessment 2005; USEPA 2006). Vascular plants in these habitats provide food, shelter, substrate, and nursery grounds for many finfish, shellfish, and wildlife (Michot and Chadwick 1994; Beck et al. 2003; Rozas and Minello 2006). Furthermore, the condition of freshwater and near-coastal ecosystems is linked to the abundance and diversity of the indigenous vegetated communities (Ziegler and Benner 1999; Deegan 2002). These communities in estuaries and associated wetlands are responsible for 43% of the estimated value of the world's ecological services (Costanza et al. 1997).

The decline of plant-dominated habitats in coastal wetlands and marshes has been documented for the Chesapeake Bay (Orth and Moore 1984; Rybicki and Carter 1986), San Francisco Bay (Zimmerman et al. 1991), Pacific

Northwest (Berry et al. 2003), and the Gulf of Mexico (Handley et al. 2007). One of the many species associated with freshwater to low-salinity areas in these habitats is *Vallisneria americana* Michx. (wild celery). It is a common submerged plant in Florida streams and lakes and is the focus of this study. *V. americana* is a valuable waterfowl food (Korschgen and Green 1988; Mitchell and Perrow 1998), is a preferred food source for manatees (White et al. 2002), and is sometimes planted for wildlife and fish habitat. This species has declined in Florida, primarily as a result of poor water quality (Jaggers 1994).

Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-*S*-triazine) is the most commonly applied restricted-use herbicide in North America (USEPA 2003a; USGS 2003) with an average annual application rate of 34 million kilograms in the USA (USEPA 2003b). Consequently, this pre- and postemergence herbicide is one of the more commonly occurring synthetic organic chemicals in freshwater and near-shore estuarine areas. As a result, its phytotoxicity to nontarget vegetation has been extensively investigated, primarily for freshwater algae and vascular species. Several risk assessments are available (for example, Solomon et al. 1996, Giddings et al. 2005), as are national acute and chronic aquatic life criteria (USEPA 2003a, b, c). Despite the relatively large database and many risk assessments, the environmental safety of atrazine continues to be an issue of debate and investigation (Demcheck and Swarzenski 2003; USEPA 2003a; Gilliom et al. 2006). Often co-occurring with atrazine and other similar herbicides (e.g., simazine) are elevated nutrient concentrations which alone commonly cause habitat deterioration and impairment of coastal rivers and estuaries (Paulic et al. 1996; USEPA 1996).

On the other hand, toxicities of most environmental contaminants in combination with common natural and anthropogenic stressors are not well understood, particularly for vascular aquatic plants (Gorsuch et al. 1991; Klaine and Lewis 1995; Klaine et al. 2002; Lewis et al. 2004). This is the case for nutrients and most co-occurring phytotoxicants. This report summarizes toxicity results for a common wetland plant which was exposed sequentially to nutrients and atrazine using chlorophyll fluorescence as an indicator of effect. This information is important for resource management and restoration of plant-dominated ecosystems and adds to the atrazine phytotoxicity database needed for future safety evaluations.

Materials and Methods

Collection and Acclimation

Whole plant cores of *Vallisneria americana* Michx. (wild celery) were obtained from the St. Johns River near Bayard Point (Clay County, FL). The 590 cores were transferred to 15-cm-diameter plastic pots and packed in 38-liter plastic tubs. The potted plants were covered with wet paper towels to prevent desiccation during delivery to greenhouse facilities at the USGS National Wetlands Research Center (NWRC, Lafayette, LA). Within 48 h of collection, the potted cores were randomly placed in 120-cm-diameter fiberglass tanks containing 76 cm deionized City of Lafayette tap water mixed with artificial seawater mix (Fig. 1). The reconstituted water was salinity adjusted (1 psu) to represent conditions at the St. John River collection site. Likewise, photosynthetically active radiation (PAR) approximated conditions at a 1 m depth in the river.

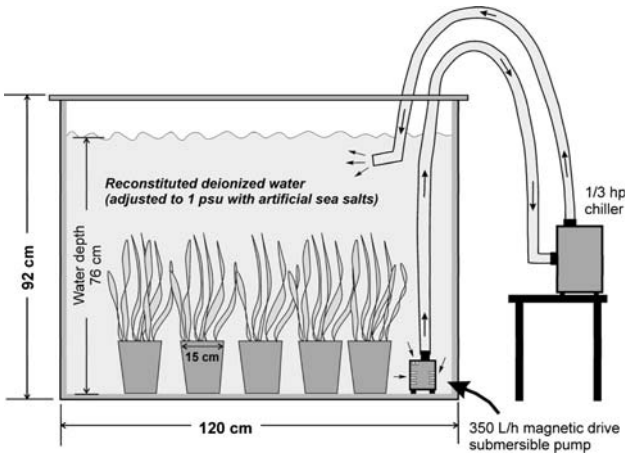

Fig. 1 Mesocosm (890-L fiberglass tank) used to sequentially expose *V. americana* to nutrients and atrazine (see Table 1 for experimental details)

Acclimation and Nutrient Pretreatment

Plants were acclimated to mesocosm conditions for 1 month prior to nutrient pretreatment for 3 months (Table 1). Potted cores of *V. americana* were effectively randomized by distributing pots from the same containers into different mesocosms until 20 had been added to each of nine recirculating mesocosms (Fig. 1). In addition to plants, each mesocosm contained juvenile sheepshead minnows (*Cyprinodon variegatus*), grass shrimp (*Palaeomonetes pugio*), and snails (*Neritina usnea*). Only the results for the plants are reported here. Each core contained an average of 12 plants. After the 1-month acclimation period, three mesocosms (randomly assigned) received low, high or ambient nutrient concentrations. The nutrient concentrations were provided by personnel from the St. Johns River Water Management District (Jacksonville, FL) for a section of the St. John River located approximately 16 km south of Jacksonville, FL. The presence of excessive nutrients and atrazine, and low dissolved oxygen are common in this large coastal river (Kroening 2004). Combined annual mean concentrations for

Table 1 Experimental conditions for acclimation, nutrient pretreatment, and atrazine exposure

Acclimation			
Mesocosms	89-L fiberglass tanks containing deionized/artificial seawater mix (0.9–1.4 psu)		
Whole-plant cores	590		
Duration	1 month		
Nutrient pretreatment			
Target nutrient loading rates (mg L^{-1} d^{-1})	NH_4^+	$NO_2^+NO_3$	PO_4^-
Low (1/3 ambient)	0.0036	0.0068	0.0028
Ambient (St. Johns River)	0.0108	0.2030	0.0084
High (3× ambient)	0.0322	0.6100	0.0253
Duration	3 months		
Temperature (°C)	18–30		
Mesocosms	Nine, 890-l recirculating fiberglass tanks		
	120 cm diameter		
	76 cm water depth		
Replicates	Three for each nutrient treatment		
Cores/test chamber	20 potted whole-plant cores		
Plants/core	Avg. 12 (±6)		
Water	Deionized water–artificial seawater mix (0.9–1.4 psu)		
Aeration	None		
Atrazine exposure			
Test duration	96 h		
Dilution water	Deionized water–artificial seawater mix (0.9–1.4 psu)		
Mesocosms	Three for each test concentration, three for controls		
Cores/test chamber	Five potted whole-plant cores		
Plants/core	Avg. 12 (±6)		
Test concentrations (μg L^{-1})	Control, 11, 110 (nominal)		
Replicates	Three each for control, 11 and 110 μg atrazine L^{-1}		
Test solution renewal	None		
Response parameter	Chlorophyll *a* fluorescence		
Temperature (°C)	23.5–27.8		
Aeration	None		

NH_4^+, $NO_3^- + NO_2^-$, and PO_4^- for this river section represented the ambient nutrient concentration. The ambient concentration was either divided or multiplied by three to form the low and high concentrations, respectively. Concentrated nutrient stock solutions were added directly to the mesocosms (Table 1) and were measured (Table 2).

During the acclimation and nutrient pretreatment periods, one-fifth of the water in each mesocosm was replaced weekly with an equal volume of deionized water artificial sea salt mix adjusted to 1.0 psu and containing the appropriate nutrient concentrations. Water in each mesocosm was continually mixed with the 350 l/h recirculating pump that routed water through an Aquanetics AFC-3B 1/3-HP chiller (Fig. 1).

The effects of the nutrients on *V. americana* were determined 70 days after nutrient addition and 5 days prior to atrazine addition. An average of 48 plants contained in four pots from each mesocosm were measured for above (leaves) and below (roots) substrate biomass (g dry wt), aerial productivity (leaf area increase, g dry wt d^{-1}), a leaf area index (Neckles et al. 1993), and epiphyte biomass (g dry wt). To provide a uniform baseline for the above-substrate biomass measurements, all leaves for each plant were clipped to approximately 15 cm above the soil line after collection and prior to placement in the mesocosm tanks.

Dissolved oxygen (mg L^{-1}), pH, temperature (°C), salinity (psu), chlorophyll *a* (mg L^{-1}), and dissolved NH_4, $NO_3 + NO_2$, and PO_4 were measured weekly in each mesocosm during nutrient pretreatment, and dissolved oxygen (mg L^{-1}), pH, temperature (°C), and salinity (psu) were measured daily during the 4-day atrazine toxicity test. Dissolved oxygen (DO), used as a secondary indicator of photosynthetic activity, temperature, salinity, and conductivity were measured with portable instrumentation. Nutrients were determined using either an Alpkem Flow Solution III autoanalyzer (O.I. Corporation, College Station, TX) or,

Table 2 Measured nutrient and atrazine concentrations in each mesocosm

Target nutrient condition[a,b]	Nutrient			Atrazine	
	NH_4^+	$NO_2 + NO_3^-$	PO_4^-	Nominal	Measured
Low (1/3 ambient)	0.008 ± 0.003	0.021 ± 0.007	0.010 ± 0.018	Control	<0.5
	0.008 ± 0.002	0.015 ± 0.009	0.007 ± 0.014	11	12.1 ± 1.1
	0.008 ± 0.003	0.017 ± 0.011	0.010 ± 0.006	110	124.0 ± 17.4[c]
Ambient (St. Johns River)	0.007 ± 0.004	0.014 ± 0.007	0.011 ± 0.026	Control	<0.5
	0.008 ± 0.002	0.016 ± 0.016	0.014 ± 0.021	11	11.1 ± 0.3
	0.007 ± 0.002	0.014 ± 0.012	0.003 ± 0.003	110	128.1 ± 10.9[c]
High (3 × ambient)	0.008 ± 0.014	0.015 ± 0.009	0.007 ± 0.014	Control	<0.5
	0.010 ± 0.005	0.021 ± 0.022	0.010 ± 0.005	11	11.4 ± 2.0[c]
	0.007 ± 0.010	0.016 ± 0.013	0.007 ± 0.010	110	107.5 ± 8.2[c]

Nutrient concentrations (mg L^{-1}) are an average (±standard deviation, SD) of nine samples taken from each mesocosm weekly during the 3-month acclimation period. Atrazine concentrations (μg L^{-1}) represent mean (±SD) of three samples from each mesocosm, 15 min after addition of stock solutions

[a] For loading rates, see Table 1

[b] Ambient values are representative of the St. Johns River

[c] Toxic effect concentrations

for NH_4, colorimetrically (USEPA 1979). Chlorophyll *a* was determined using a modified procedure of Wetzel and Likens (1991) and a Turner Fluorometer 10AU (Turner Designs Corp., Sunnyvale, CA). Weekly PAR measurements were taken at the surface and at 37 cm depth using a LICOR spherical sensor and handheld display.

Atrazine Toxicity Test

Nine mesocosms were used for the atrazine toxicity test (Table 2). Three plant-containing mesocosms (one each for the three nutrient pretreatments) were exposed to nominal 0, 11 or 110 μg l^{-1} atrazine for 96 h. The two nominal atrazine test concentrations were added once to the mesocosms and originated from a concentrated (180 ppm) stock solution (source, USEPA Standard Repository, Fort Meade, MD, lot #5961870). The test concentrations were measured at time of test initiation for triplicate samples collected from each mesocosm using an enzyme-linked immunosorbent assay (ELISA) technique (EnviroGuard® triazine plate kit #72110; Strategic Diagnostics Inc., Newark, DE). See Table 2 for results.

A pulse-amplitude modulated (PAM) fluorometer (Heinz Walz GmbH, Inc., Effeltrich, Germany) was used to measure chlorophyll *a* fluorescence, which serves as an indication of the percentage of light energy used by the photosystem II reaction of photosynthesis (Schreiber 2004). It is often reported as electron transport rate or ETR which provides an "approximation of the rate of electrons pumped through the photosynthetic chain" (Ralph and Gademan 2005). Calculations of ETR are known to provide an indication of photosynthetic efficiency and have been shown to be closely parallel oxygen evolution and CO_2 uptake (Beer et al. 1998). In general, high fluorescence indicates low photosynthetic activity (Schreiber 1997). Measurements were made between 12:00 and 14:00 h at 0, 24, 48, and 96 h from five-second-order leaves (second oldest from rosette center) randomly selected from the plants in each mesocosm. During the initial readings (i.e., 0 h, pre-exposure), loose-fitting color-coded plastic paper clips (2.5 mm length) were first positioned on each selected leaf above the point of measurement to insure that the same position on each replicate leaf was used during successive measurements.

Maximum electron transport rates (ETR) values for each leaf were obtained using a nine-point rapid light curve (RLC) technique (Ralph et al. 1998; Schreiber 2004; Ralph and Gademan 2005) that reduces the need to perfectly maintain environmental conditions (e.g., temperature, ambient light, CO_2 concentration) over time (Heinz Walz GmbH 1998). Specially designed "dark" leaf clips (Optic-Sciences, Inc., Tyngsboro, MA) were first attached to the leaves at approximately 0.5 cm below each of the plastic clips. The clips were used to ensure a consistent distance of 10 mm between the leaf tissue and the combined fiber-optic sensor and actinic light source instrument probe. Neoprene contact points built into the clips prevented interference from extraneous sources of PAR. Using the RLC mode on the PAM instrument, ETRs were calculated based on measures of quantum yield at 10-s intervals using a preset series of nine PAR irradiance intensities (0, 43, 97, 167, 566, 391, 584, 853, and 1,255 μmol photons m^{-2} s^{-1}) generated by an internal halogen lamp. The RLC measurement procedure was performed for each of the five

clipped leaves at random within each mesocosm 6–12 min after attachment of the leaf clips.

Data Analysis

Maximum ETR values were compared for plants exposed to atrazine relative to those in controls and also between the three nutrient pretreatments and each of the two atrazine test concentrations. Statistical analysis was conducted using a multivariable repeated measure analysis of variance (ANOVA)(SAS Institute, Inc., Cary, NC) which controls for correlation between measurements on the same subject (leaf) taken repeatedly over time by treating each sampling event as a separate response variable. The Latin square design tested the effects of nutrients, atrazine, and the interaction, while controlling for repeated measures. A one-way ANOVA was used to compare the DO between controls and combined nutrient–atrazine treatments to serve as a secondary indicator of photosynthetic inhibition.

Results

Nutrient Pretreatment

Water temperature, salinity, pH, dissolved oxygen, and PAR varied less than 10% for the low, ambient, and high nutrient pretreatment mesocosms. Water temperature during nutrient pretreatment averaged 26.9 ± 2.1°C (range 20.0–29.9°C). Mean salinity was 1.0 ± 0.1 psu (range 0.9–1.2 psu) and dissolved oxygen ranged from 6.3 to 9.8 mg L^{-1} (mean 8.0 mg L^{-1}). pH was between 7.5 and

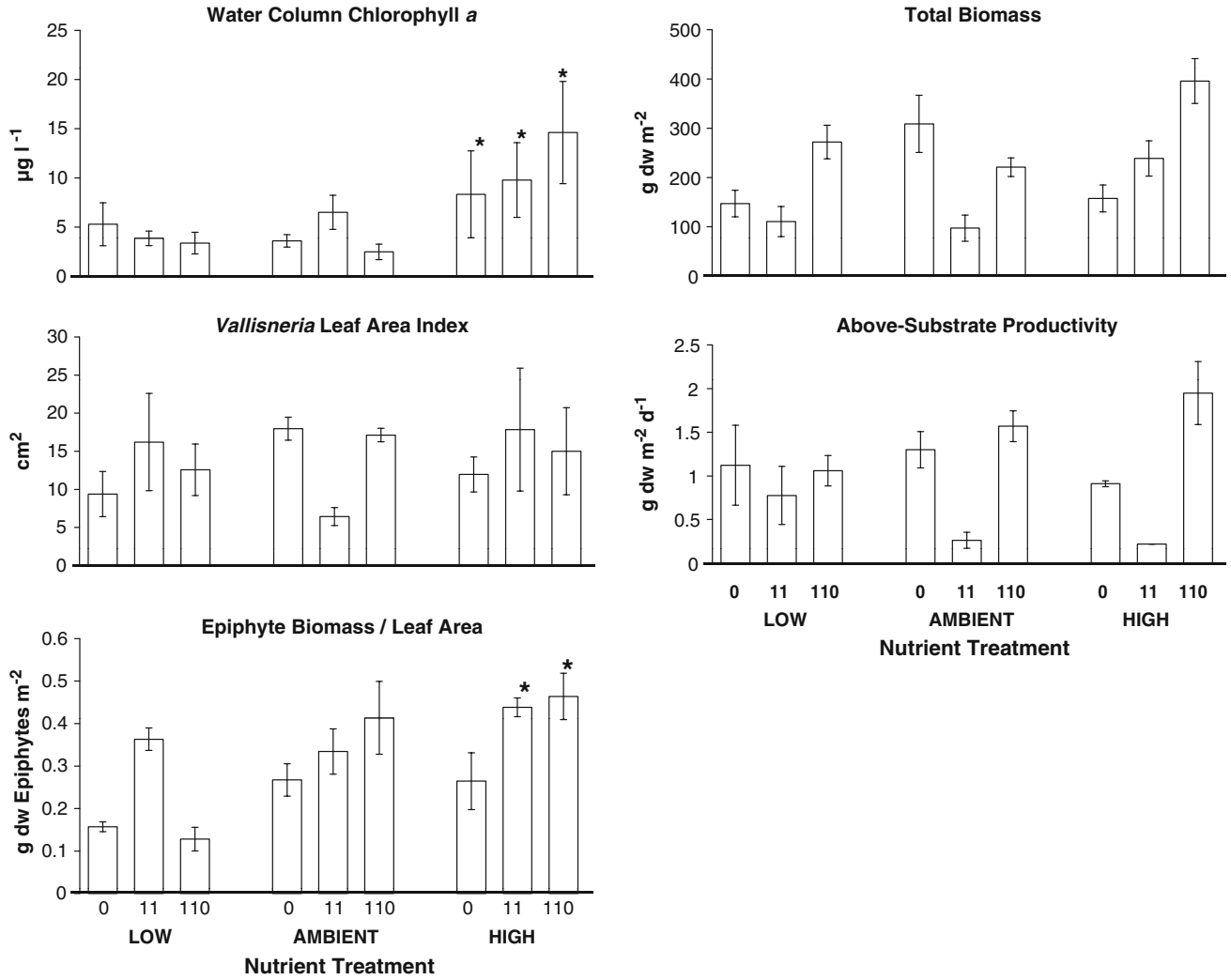

Fig. 2 Chlorophyll *a*, epiphyte, and plant structural characteristics after nutrient pretreatment period (3 months) and 1 week prior to atrazine exposure. Results represent means (standard error, SE) of three replicate mesocosms for each nutrient treatment. Labels "0," "11," and "110" beneath bars indicate the atrazine dose applied following nutrient pretreatment. See Tables 1 and 2 for nutrient loading rates and measured concentrations. Leaf area index from Neckles et al. (1993)

9.6. PAR at the surface averaged 753.7 ± 136.1 µmol quanta/m^2/s and 516.4 ± 144.2 µmol quanta/m^2/s at 37 cm.

Nutrients alone impacted *V. americana* (Fig. 2). Chlorophyll *a* and epiphyte biomass were significantly greater for plants exposed to the high nutrient concentration ($p < 0.05$) (Fig. 2). No significant differences occurred for the leaf area index and whole plant biomass. Rapid reduction of nutrient availability (Table 2) during pretreatment, regardless of concentration, was obvious and consistent with those of other nutrient-related studies (Twilley et al. 1985, Boustany et al. 2000).

Atrazine Exposure

Significant decreases in ETR values occurred for nutrient-pretreated plants after 24, 48, and 96 h exposure to the nominal 110 µg L^{-1} atrazine test concentration (range of mean measured concentrations 107.5–128.1 µg L^{-1}) (Fig. 3). Reductions also occurred at 11 µg L^{-1} (mean measured 11.4 µg L^{-1}) for plants pretreated only with the high nutrient concentration. The maximum ETR values from each rapid light curve (RLC) procedure were averaged to show differences in ETR responses to the two atrazine test concentrations (Fig. 4). Obvious differences in ETR values occurred between control plants and all nutrient-pretreated plants exposed to nominal 110 µg L^{-1} atrazine. Only at the high nutrient pre-exposure did separation between ETR values for plants in the controls and those exposed to 11 µg L^{-1} occur. For example, after 96 h, decreases of 46% relative to control were observed. In general, ETR responses for ambient and low-nutrient-pretreated plants exposed to this concentration approximated the RLC of the controls.

Measurements of dissolved oxygen were lower in the water column of the atrazine-exposed plants relative to values for control plants (Fig. 5), further reflecting impairment of photosynthetic activity. A maximum reduction of 24% (dissolved oxygen) occurred for plants exposed to nominal 110 µg L^{-1} atrazine after pre-exposure to the high nutrient concentrations. Reductions generally paralleled the decreases in ETR activity.

Discussion

Environmental modification of chemical toxicity is an important consideration in the hazard assessment process for chemical registrations and site-specific risk evaluations. Several natural and anthropogenic factors such as the presences of nutrients, as reported herein, can affect phytotoxicity to vascular plants as well as exposure duration. Significant interaction ($F = 9.95$, $p = 0.0034$) occurred

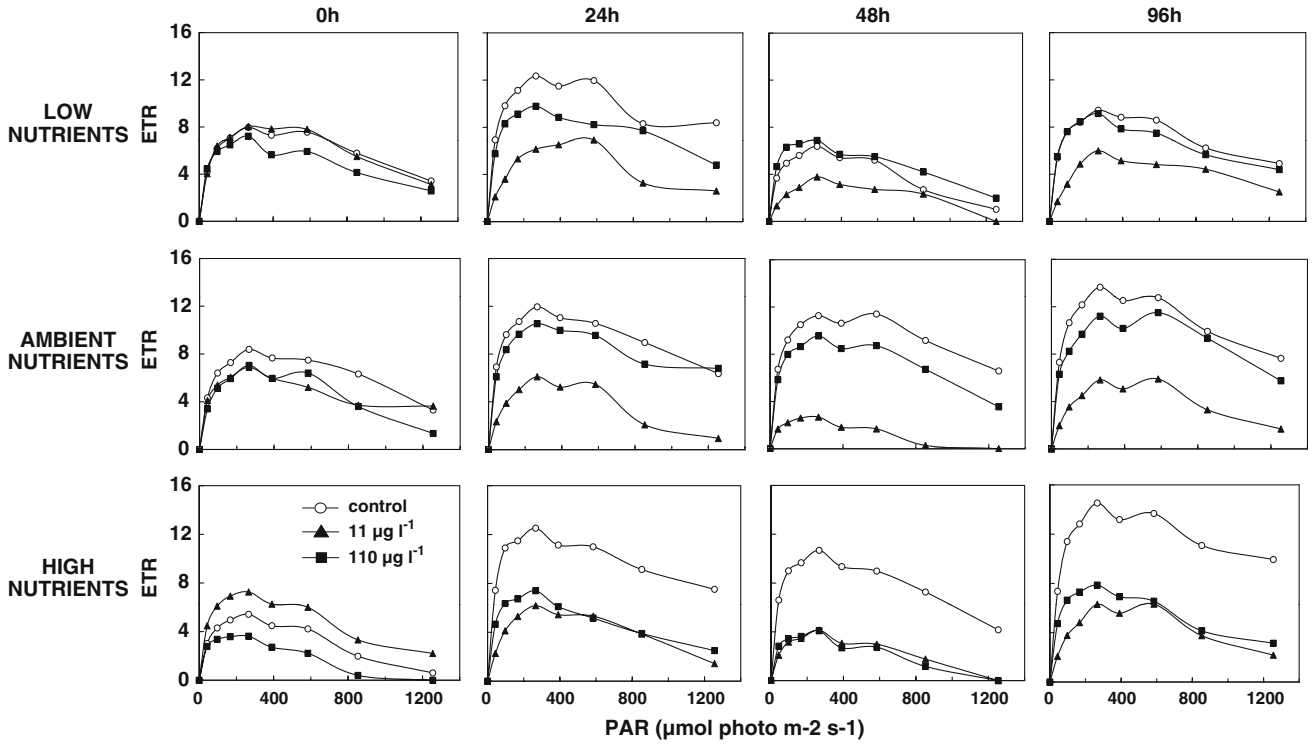

Fig. 3 Electron transport rates (ETR) for *V. americana* exposed to atrazine for 96 h. Values represent the mean of five-nine-point rapid light curves. Photosynthetically active radiation (PAR) at 400–700 nm and preset intensities of 0, 43, 97, 167, 566, 391, 584, 853, and 1,255 µmol photons m^{-2} s^{-1}

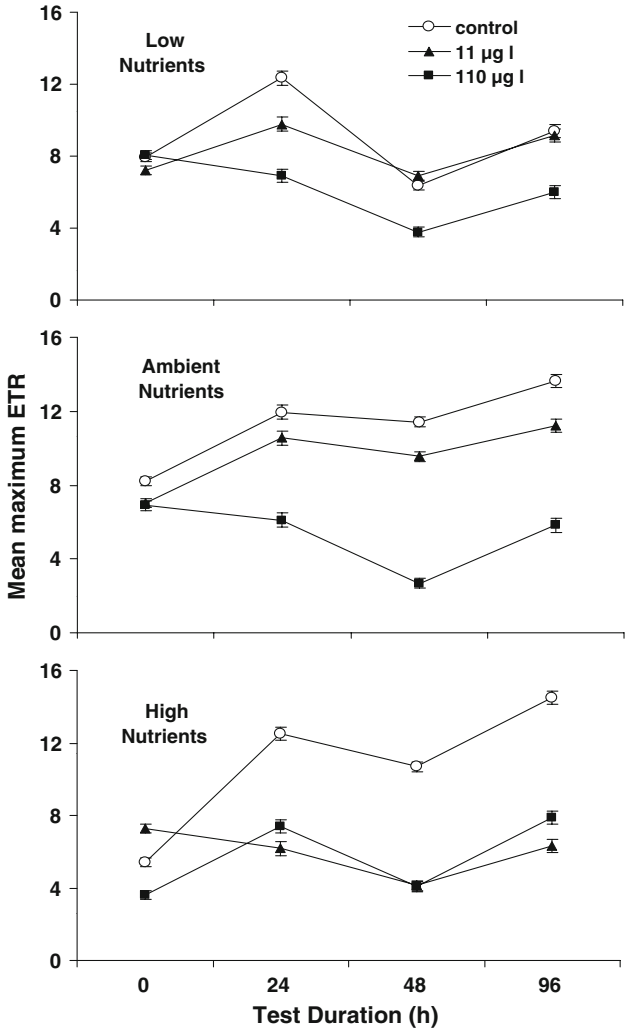

Fig. 4 Maximum electron transport rates (ETR) for *V. americana* exposed to atrazine after nutrient pretreatment. Values represent means (±SE). Measured nutrient and atrazine concentrations in Table 2

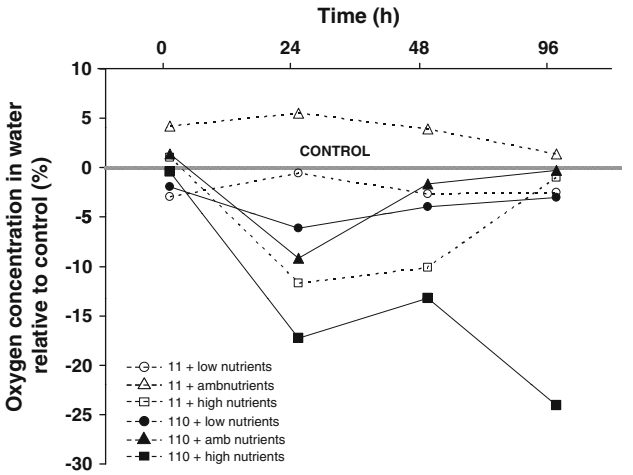

Fig. 5 Percent differences for mid-day dissolved oxygen (mg L^{-1}) during the 96-h toxicity test (see Table 2 for measured nutrient and atrazine concentrations)

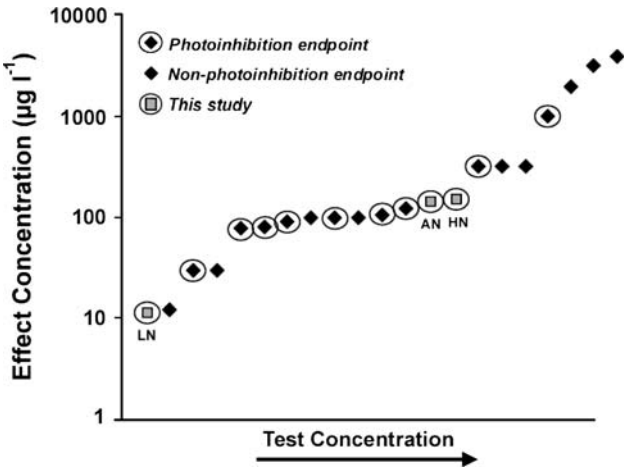

Fig. 6 Comparison of atrazine phytotoxicity as reported here with previously reported toxic effect concentrations. Other results are for eight vascular plant species evaluated under conditions of different salinities (3–30 psu) and test durations (2–47 days). *LN* low nutrient pretreatment, *AN* ambient nutrient pretreatment, *HN* high nutrient pretreatment; figures adapted from USEPA (2003b)

between the concentrations of nutrients and atrazine and between toxicity and test duration ($F = 25.21$, $p = 0.0004$).

The mean measured effect levels reported here of 107.5–128.1 µg L^{-1} atrazine (for all nutrient treatments) and 11.4 µg L^{-1} (high nutrient treatments only) are within the range of previously reported phytotoxic concentrations for this herbicide and various vascular plants (Fig. 6). For example, lowest observed effect concentrations (LOEC) reported for atrazine and wetland species such as *Vallisneria americana*, *Potamogeton spp.*, and *Myryriophyllum spicatu,* have ranged from 12 to 320 µg L^{-1} (USEPA 2003c). Atrazine concentrations as low as 30 µg L^{-1} reduced photosynthetic activity of *Vallisneria gigantean* after 21 days exposure (Hohnberg et al. 2003). The lowest effect concentration of 11.4 µg L^{-1} from our study, however, is at the lower end of the range of reported phytotoxic concentrations and is less than the national saltwater chronic criterion of 17 µg L^{-1} (USEPA 2003c). The level of concern for the aquatic ecosystem monitoring program related to the atrazine re-registration process is 10–20 µg L^{-1} (www.epa.gov/pesticides/reregistration/atrazine).

The presence of atrazine is a concern in the St. Johns River (Boyce et al. 2003); the few reported concentrations have averaged 0.18 ± 0.27 µg/l (range below detection–0.77 µg/l). Comparison of the mean and maximum environmental levels to the lowest effect level determined in this study (11.4 µg L^{-1}) results in safety factors of at least 63 and 15, respectively, for *V. americana*, suggesting a lack of impact. The relatively wide safety margin (15 or

greater) suggests a lack of impact on *V. americana*, but this assessment is preliminary and needs refinement. It assumes that all plant life stages of this species are equally sensitive, that chlorophyll *a* fluorescence is a sensitive and relative indicator of effect, that there are no other phytotoxicants present, and that there is a steady-state ambient atrazine concentration. Atrazine is a reversible inhibitor of photosynthetic activity. This latter assumption is unlikely since atrazine has been shown to commonly occur in combination with metolachlor, prometon, and simazine in south Florida surface waters (McPherson et al. 2000). Therefore, in situ studies should be conducted to confirm our conclusion.

Finally, our experimental design is worth consideration to develop first-phase toxicity tests for phytotoxicants and nontarget plants. In situ measurement of pulse-amplitude modulated (PAM) fluorometry was a rapid and consistent indicator of photosynthetic stress as it has been elsewhere (Ralph et al. 1998; Maxwell and Johnson 2000; Beer et al. 2001; Baker and Oxborough 2004; Ralph and Gademan 2005). The nine-point rapid light curve (RLC) technique (Schreiber 2004; Ralph and Gademan 2005) provided efficient and reliable measures of ETR. In most cases, we were able to complete RLC measurement cycles for five *V. americana* leaves in less than 12 min. The resulting database provided enough degrees of freedom to statistically detect important differences and interactions.

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