Journal of Hazardous Materials 191 (2011) 356–365

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Comparison of morphology and photo-physiology with metal/metalloid contamination in *Vallisneria neotropicalis*

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ARTICLE INFO

Article history:
Received 25 November 2010
Received in revised form 19 April 2011
Accepted 20 April 2011
Available online 29 April 2011

Keywords:
Submerged aquatic vegetation (SAV)
Pollution
Gulf of Mexico
Effective quantum yield of photosystem II (PSII
$\Delta F/F_m'$)
Photosynthetic performance

ABSTRACT

The overarching goal of this *in situ* study was to investigate the integrated impact(s) that metal/metalloid contamination might have on the overall health and performance of the ecologically important aquatic macrophyte, *Vallisneria neotropicalis*. Morphological (i.e., shoot growth-based endpoints) and photo-physiological (i.e., photosynthetic activity measured as chlorophyll *a* fluorescence and oxygen exchange) variables, along with aboveground tissue metal/metalloid concentrations, were measured in natural populations of *V. neotropicalis* that differed with respect to their anthropogenic pressure. With the exception of an overall negative effect on growth, our results suggest that there were no detrimental effects of low/moderate contamination of *V. neotropicalis* by trace elements (i.e., arsenic As and mercury Hg; 1.04–2.77 $\mu g\,g^{-1}$ dry wt. and 3.76–15.18 $ng\,g^{-1}$ dry wt., respectively) on the photosynthetic physiological performance of this species. *V. neotropicalis* appears to tolerate low/moderate levels of trace element contamination with little impact on plant health and performance.

1. Introduction

Vallisneria americana Michaux is an aquatic angiosperm that forms extensive meadows in freshwater to mesohaline environments [1]. These meadows play important ecological roles, i.e., they constitute the basal food resource for complex and prolific trophic webs [1–6], are significant intermediaries in the cycling of carbon and nutrients in the ecosystem [7,8], promote sedimentation and enhance sediment stability [7], and provide shelter and nursery habitats for numerous recreationally and economically-important species of shellfish and finfish [9–11]. *V. americana* is distributed from Central America to Canada [4]. However, morphological and molecular characters differ among northern and southern populations (i.e., well-described ecotypes exist [12–14]). A recent study of the genus *Vallisneria* recognizes the Gulf Coast species, *Vallisneria neotropicalis* Marie-Vict., as being phenotypically and genetically distinct from *V. americana* [15].

Over the past 50 years, worldwide declines in macrophyte abundance and distribution have been reported in freshwater, estuarine, and marine environments [16–22]. Such large-scale losses among aquatic plant communities, including those dominated by *Vallisneria* species, have been linked to human development of coastal watersheds, and subsequent increases in anthropogenic pollution and urban runoff [8,16,17,20,21,23–26]. Anthropogenic chemicals (e.g., trace metals, polycyclic aromatic hydrocarbons or PAHs, and pesticides) appear to be particularly noxious/toxic to aquatic plants. Indeed, reductions in growth and/or photosynthetic performance have been observed in several species following exposure to various chemical treatments (e.g., *Halophila ovalis*: [31–33], *Myriophyllum spicatum*: [27,28], *Ruppia maritima*: [29], *Zostera marina*: [30], *Z. capricorni*: [31–33]).

Aquatic plants have been extensively studied as model bioaccumulators of various xenobiotics; their potential as biomonitors of organic and inorganic contamination in aquatic ecosystems has been investigated worldwide (see reviews [38–40]). Ironically, despite their ecological importance, few studies have addressed the potential biological effects of xenobiotic contamination on these organisms (see reviews [37,41]). Moreover, the majority of previous investigations involve the measurement of plant responses following xenobiotic exposure under controlled laboratory conditions (e.g., [32,42,43]), and do not necessarily represent or take into account the complexity of natural systems [44,45]. For instance, Macinnis-Ng and Ralph [46] noted that laboratory experiments significantly overestimated the impact(s) of some herbicides on the seagrass, *Z. capricorni*, relative to field experiments. Thus, field-based studies are important for the assessment of potential long-term impacts of contaminants on aquatic plant health and

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doi:10.1016/j.jhazmat.2011.04.091

performance. This is especially true for freshwater and estuarine species, since most studies to date have targeted marine species (e.g., *H. ovalis*: [32,33,43] – *Z. capricorni*: [34,43,47]).

The overarching goal of this study was to investigate the integrated impact(s) that trace element contamination might have on overall plant performance among natural populations of the ecologically important plant, *V. neotropicalis*. For this purpose, we compared morphological and photo-physiological attributes, along with the metal/metalloid levels in aboveground tissues, in natural populations of *V. neotropicalis* that are exposed to different levels of anthropogenic pressure.

2. Material and methods

2.1. Study area

The study area is part of the Mobile Bay Estuary System, a coastal transition zone between the Mobile Bay watershed and Gulf of Mexico (Alabama, USA; Fig. 1). The Mobile Bay watershed drains two-thirds of the state of Alabama and portions of Mississippi, Georgia, and Tennessee. It is the fourth largest watershed in terms of freshwater discharge volume (1756 $m^3 s^{-1}$ on average), and the sixth largest river system with respect to total area (draining 113,084 km^2) in the United States [48]. The study area includes: (i) the lower Mobile-Tensaw Delta, on the northern shore of Mobile Bay, formed by the merging of the Alabama and Tombigbee Rivers at the terminus of the Mobile River Basin [49] and (ii) the Fish River, on the eastern shore of Mobile Bay, that accounts for approximately 73% of the freshwater inflow into Weeks Bay ([50]; Fig. 1).

Estuaries of the southern coast of the United States are particularly threatened by trace element contamination, specifically by As (a metalloid) and Hg (a metal) contamination. In 2007, the highest concentrations of total Hg wet deposition in the United States were found off the Gulf Coast [51]. The entire coastline of the Gulf is under a Hg advisory [52], and several of its waterbodies are included on the US Clean Water Act 303(d) list as being impaired with respect to Hg and/or As [53]. As and Hg are rated among the most toxic metals/metalloids. They rank first and third, respectively, on the 2007 CERCLA (Comprehensive Environmental Response, Compensation, and Liability Act) priority list of hazardous substances [54]. Pollution of the environment by As and Hg poses serious environmental and human health concerns on a worldwide scale [55–58].

2.2. Sampling sites

A total of six sampling sites were chosen for this study. Four sampling sites were set up in the lower Mobile-Tensaw Delta (Delvan Bay DB: 30°42′681″N, 88°00′700″W – Chacalochee Bay CB: 30°40′543″N, 87°58′048″W – Meaher Park MP: 30°39′899″N, 87°57′096″W – Bay Minette BM: 30°41′878″N, 87°55′420″W); two additional sites were set up along the Fish River in the Weeks Bay National Estuarine Research Reserve (WBNERR; Water Hole WH: 30°25′895″N, 87°49′595″W – Turkey Branch TB: 30°25′788″N, 87°49′729″W – Fig. 1).

These sites differ with respect to anthropogenic impact. The north-western side of Mobile Bay is highly urbanized and industrialized relative to the eastern side that is nearly undeveloped. Indeed, the city of Mobile, the second largest metropolitan area in the state of Alabama (2007 population estimate: 404,097; [59]), is located on the northwest edge of Mobile Bay (Fig. 1). Additionally, the Warrior Field, that accounts for most of the coal production in Alabama [60] and ranks 15th among coal-producing states in the US [61], is located in the northwestern quarter of the Mobile Bay watershed; the coal mined from the Warrior Field is unusually enriched in As and Hg [60,62,63].

2.3. In situ measurements and sampling

2.3.1. Physico-chemical site characterization

In situ measurements of depth, ambient water temperature, salinity, dissolved oxygen (YSI©– 85 handheld DO/Conductivity Instrument), pH (YSI©– Environmental EcoSense handheld pH100), and light attenuation (Li-COR©– LI-193 underwater spherical quantum sensor connected to a LI-1400 datalogger) were made at each site throughout July and August 2008. Dissolved oxygen (DO) measurements were made at 0 (DO-s; s: surface) and 0.5 m depth (DO-d; d: depth). Water samples were collected in triplicate at mid-water column from each site for the measurement of water column nutrient (dissolved inorganic nitrogen or DIN = NO_2^- + NO_3^-, and ammonium NH_4^+) and chlorophyll *a* concentrations. Samples for DIN, were field-filtered, placed on ice, and frozen for later analysis following the method described by Eaton et al. [64]. To minimize degradation effects, samples for NH_4^+ analysis were also field-filtered, placed on ice, and immediately processed upon return to the laboratory as described by Parsons et al. [65]. Chlorophyll *a* was quantified spectrophotometrically (Perkin-Elmer©– Lambda 4A UV/Vis spectrophotometer) following extraction with 2.5 mL N,N-dimethylformamide (DMF) as described by Dunton and Tomasko [66] and applying the equations of Porra et al. [67].

2.3.2. Abundance of V. neotropicalis and other plant species

Transects (10 m × 20 m), running parallel to the shore, were set up in each of six sampling sites (Fig. 1). Percent cover per species was visually determined at 5-m intervals in each of 12 (1 m × 1 m) quadrats, both along the shore and perpendicularly outward to a depth of 2 m, yielding estimates of abundance and frequency of occurrence [68]. For the purposes of this study, presence was defined as the occurrence of a stem or plant within a quadrat (i.e., stems/plants were rooted or floating in the observation area).

2.3.3. In situ chlorophyll a fluorescence measurements

The effective quantum yield of photosystem II (PSII; $\Phi_{PSII} = (F_m' - F_s)/F_m' = \Delta F/F_m'$; [69]) was measured under ambient light conditions with a diving-PAM (Pulse Amplitude Modulated) underwater fluorometer (Heinz Walz GmbH©). To standardize fluorescence measurements, leaves were measured and data were consistently gathered from mid-way between the basal meristem and leaf tip of the longest leaf of each replicate plant (n = 20 plants/site per sampling date). Leaf sections used for fluorescence measurements were subsequently collected and placed on ice for later extraction and pigment quantification. With the exception of the Delvan Bay population which was only sampled twice (n = 40 total), weekly fluorescence data were collected from each population/site over the month of July 2008 (i.e., *V. neotropicalis* populations were sampled four times over the study period, n = 80 total per site). While we recognize that light intensity necessarily affects photosynthetic performance and the fluorescence yield [70,71], logistical constraints precluded gathering all data points (from all sites) at the same time of day. To minimize the effect of variation in light intensity, sites were visited four times over the study period (as mentioned above) in random order. All measurements were conducted between 8.30 a.m. and 13.30 p.m. per sampling date and data was then pooled for each sampling site to compare mean fluorescence yield among sites over the study period.

2.3.4. Collection of V. neotropicalis samples

V. neotropicalis plants were carefully hand-extracted from sediments for subsequent morphological, physiological, and trace metal measurements. Plants collected for morphometric analyses were placed in bags on ice, transported to the laboratory, and frozen for later processing. Plants used for photosynthetic measurements

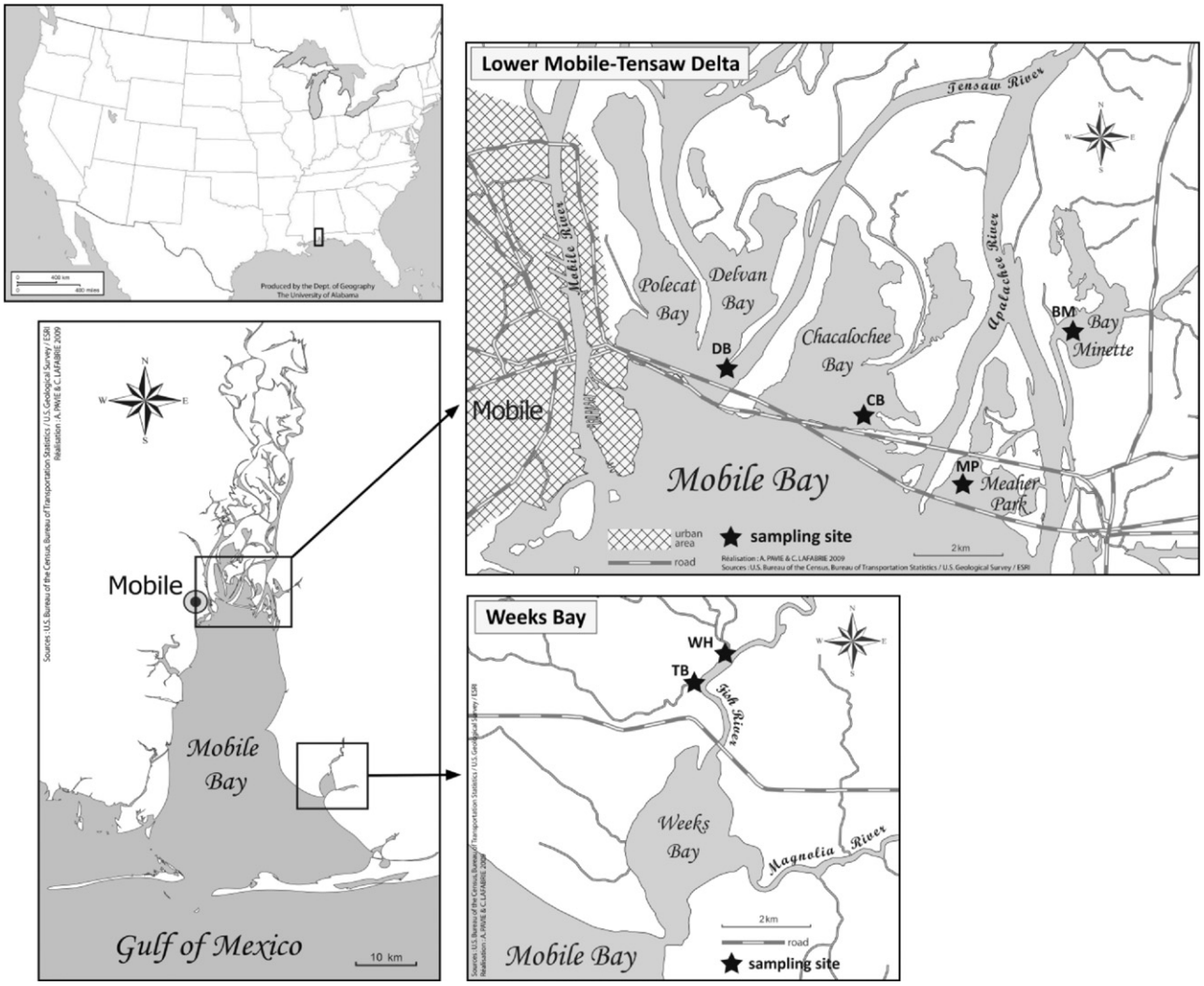

Fig. 1. Study area and study sites (DB: Delvan Bay, CB: Chacalochee Bay, MP: Meaher Park, BM: Bay Minette, WH: Water Hole, TB: Turkey Branch).

were carefully transported to the laboratory in seawater-filled buckets equipped with battery-operated air pumps for immediate processing. Plants collected for trace metal analyses were placed in bags on ice, and transported to the laboratory. Once at the laboratory, leaves were carefully washed, cleaned of sediment and periphyton with ultra-pure water, placed in sample bags, and frozen. Once frozen, samples were freeze-dried (6L Labconco Console FreeZone Freeze-Dry system from Labconco Corp.), manually ground to a coarse powder, and stored for later analysis.

2.4. Laboratory measurements

2.4.1. Photosynthetic pigment content

Once at the lab, leaf sections used for fluorescence measurements were blotted dry with paper towel, weighed, and cut into thin sections with a straight-edged razor for efficient extraction. Each sample was placed in a glass, screw-capped vial with 2.5–5.0 mL N,N-dimethyl-formamide (DMF) as described by Dunton and Tomasko [66]. Samples were placed in the dark and allowed to extract at room temperature for seven days prior to spectrophotometric determination of pigment content using a Perkin-Elmer©– Lambda 4A UV/Vis spectrophotometer. Absorbance was measured at 480, 510, 646.8, 663.8 and 750 nm; absorbance at 750 nm was subtracted from absorbance values at all other wavelengths to correct for scattering due to turbidity. Chlorophyll *a* and *b* concentrations were calculated using the equations provided by Porra et al. [67]. "Bulk" carotenoid concentrations were estimated using the equations of Parsons et al. [65] after Henley and Dunton [72].

2.4.2. Photosynthetic measurements

V. neotropicalis plants from each site were maintained in aquaria filled with aerated ambient seawater, and placed under a bank of cool fluorescent white lights (*ca.* 150–200 μmol photons m^{-2} s^{-1}, saturating for photosynthesis; [73,74]). To standardize photosynthetic measurements, a 1 cm-long section was cut from the longest leaf, mid-way between the basal meristem and leaf tip, of each replicate plant. Leaf segments were gently cleaned with a paper towel, weighed, and allowed to wound-respire in the dark for ~20 min prior to use. Photosynthetic measurements were made using an automated, temperature-controlled Hansatech OxyLab system, as previously described (see [66,75,76]). The incubation chamber contained 2.5 mL filter-sterilized ambient seawater spiked with $NaHCO_3^-$ to achieve a final concentration of 8.0 mM. Samples were maintained at a temperature of 30.5 °C (i.e., ambient field temperature) and incubated for 15 min at each irradiance level (0 and 500 μmol photons m^{-2} s^{-1}). Rates of dark respiration (R_d) and light-saturated photosynthesis (P_{max}) were normalized to fresh weight. Values for P_{max} represent net oxygen evolution.

Table 1
Physico-chemical site characteristics (mean ± SE, $n = 3$ except for DB where $n = 1$).

	DB	CB	MP	BM	WH	TB
Depth (m)	0.5	0.6 ± 0.1[a]	0.5 ± 0.1[a]	1.0 ± 0.1[a]	1.0 ± 0.1[a]	0.7 ± 0.1[a]
Temperature (°C)	31.1	30.3 ± 0.9[a]	29.8 ± 1.3[a]	31.4 ± 1.2[a]	30.0 ± 1.0[a]	30.1 ± 1.0[a]
Salinity (‰)	4.3	0.7 ± 0.4 [a]	1.7 ± 0.9 [a]	1.0 ± 0.6 [a]	0.9 ± 0.5 [a]	0.9 ± 0.5 [a]
DO-s (mg L^{-1})	8.4	5.1 ± 0.7[a]	7.9 ± 0.5[a]	6.6 ± 1.4[a]	6.3 ± 0.4[a]	7.7 ± 0.4[a]
DO-d (mg L^{-1})	8.2	5.5 ± 0.6[a]	6.9 ± 0.2[a]	5.8 ± 1.2[a]	5.7 ± 0.3[a]	8.0 ± 0.0[a]
pH	7.9	7.7 ± 0.1[a]	8.0 ± 0.3[a]	7.7 ± 0.1[a]	7.0 ± 0.1[a]	7.3 ± 0.4[a]
DIN (NO_2^- + NO_3^-; μM)	0.3	0.2 ± 0.1[a]	0.1 ± 0.1[a]	0.2 ± 0.1[a]	3.3 ± 1.0[a]	3.6 ± 1.6[a]
NH_4^+ (μM)	0.0	0.9 ± 0.5[a]	0.1 ± 0.1[a]	1.4 ± 1.4[a]	2.4 ± 1.2 [a]	2.4 ± 1.6[a]
Chl *a* (nM)	25.5	15.8 ± 1.5[a]	9.7 ± 1.8[a]	15.7 ± 0.9[a]	32.4 ± 18.4[a]	40.0 ± 18.6[a]

DB: Delvan Bay, CB: Chacalochee Bay, MP: Meaher Park, BM: Bay Minette, WH: Water Hole, TB: Turkey Branch. DO: dissolved oxygen; s: surface; d: depth; DIN: dissolved inorganic nitrogen; Chl *a*: chlorophyll *a*.
Means sharing the same letter do not significantly differ ($P \geq 0.05$).

2.4.3. Morphological measurements

Shoot height was determined as the distance from the base of the stem to the tip of the longest leaf. The number of leaves on each shoot was counted. The aboveground and belowground biomass were separated, dried at 60 °C to a constant weight, and weighed to the nearest 0.001 g.

2.4.4. Trace element analysis

Prior to As analyses, samples (i.e., coarse powders of *V. neotropicalis* leaves) were digested with nitric acid (HNO_3, reagent grade) and hydrogen peroxide (H_2O_2, reagent grade) in tubes heated to 95 °C. As concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS), using the US EPA Method 6020 [77]. Hg concentrations were determined with a Hydra-C DMA (Teledyne Leeman Labs), instrument employing the "thermal decomposition–amalgamation–atomic absorption spectrophotometry" process (US EPA Method 7473; [78]). The certified reference materials BCR-060 (*Lagarosiphon major*, Community Bureau of Reference – Commission of the European Communities) and TORT-2 (lobster hepatopancreas, National Research Council Canada) were routinely digested and analyzed alongside field-collected samples to determine the recovery rate of trace elements and verify the analytical procedure (As recovery for TORT-2 = 90% and Hg recovery for BCR-060 = 94%).

2.5. Statistical analysis

Differences in environmental variables, *V. neotropicalis* morphometric and photosynthetic characteristics, and As and Hg leaf concentrations among sites were determined using STATISTICA™'s Kruskal–Wallis analysis of variance (ANOVA). This test was used because assumptions of normality and homogenous variance could not always be met. Where Kruskal–Wallis ANOVA revealed significant differences, Student–Newman–Keuls multiple comparisons were performed. Multiple regression analysis was used to determine if tissue concentrations of As and/or Hg in *V. neotropicalis* were correlated with measured morphometric and photosynthetic parameters of the plant. All tests were assessed at the $P = 0.05$ level.

3. Results

3.1. Environmental parameters

Environmental parameters did not vary with site. Although nutrient and chlorophyll *a* concentrations were higher in Weeks Bay relative to the Mobile-Tensaw Delta, such trends were probably due to substantial within-site variability (Table 1).

The six study sites were largely dominated by the native species, *V. neotropicalis* and *Najas guadalupensis*, with values for percent cover ranging from 30 to 72 and 10 to 54%, respectively (Table 2). Bay Minette was the only site characterized by a large non-native plant component; *M. spicatum* cover was estimated to be *ca.* 23% in this population. Although no measurement of percent cover was made in Delvan Bay, the macrophyte population was extensive and also dominated by *V. neotropicalis* and *N. guadalupensis*.

3.2. Morphological variables (*V. neotropicalis*)

V. neotropicalis shoots were tallest in Bay Minette (80.8 ± 4.9 cm) and significantly different from all other populations, excepting the Delvan Bay site (Fig. 2A). Conversely, the shortest plants occurred in Meaher Park (31.0 ± 1.4 cm) and the two Weeks Bay sites (24.8 ± 1.1 cm, WH and 25.5 ± 0.9 cm, TB; Fig. 2A). Few significant differences were noted among sites with respect to the number of leaves per plant; plants growing in Turkey Branch and Delvan Bay had more leaves than those in Bay Minette (10.2 ± 0.4 and 9.7 ± 0.4 *cf.* 7.3 ± 0.5 leaves $shoot^{-1}$; Fig. 2B). Values for both above- and below-ground biomass were greater in Bay Minette, Delvan Bay, and Chacalochee Bay when compared to all other populations sampled. *V. neotropicalis* above-ground biomass did not significantly differ between Meaher Park and Chacalochee Bay, while below-ground biomass did not significantly differ among Meaher Park, Chacalochee Bay, and Delvan Bay (Fig. 2C). With the exception of

Table 2
Percent cover of native (*Vallisneria neotropicalis*, *Najas guadalupensis*, *Juncus romerianus*) and non-native (*Myriophyllum spicatum*, *Potamogeton crispus*) plant species.

	Vallisneria neotropicalis	*Najas guadalupensis*	*Juncus romerianus*	*Myriophyllum spicatum*	*Potamogeton crispus*
DB	ND	ND	ND	ND	ND
CB	34	24	–	–	1
MP	30	54	–	–	–
BM	48	15	6	23	–
WH	61	51	–	–	–
TB	72	10	–	–	–

DB: Delvan Bay, CB: Chacalochee Bay, MP: Meaher Park, BM: Bay Minette, WH: Water Hole, TB: Turkey Branch.
ND: No Data.
–: absence of the species considered.

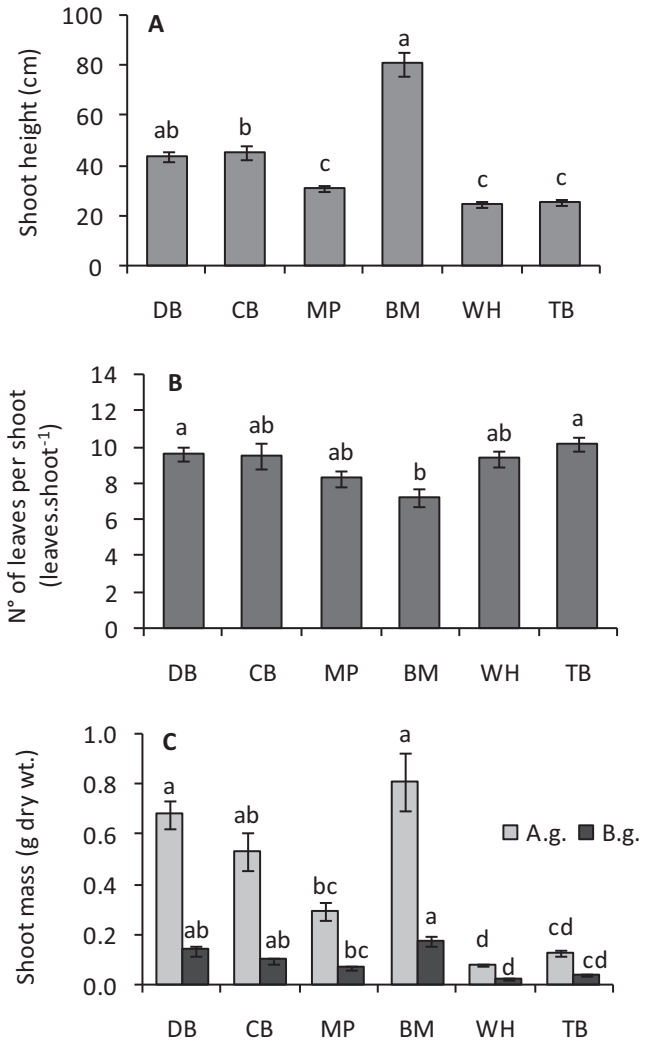

Fig. 2. *Vallisneria neotropicalis*. (A) Shoot height (cm), (B) number of leaves per shoot (leaves shoot^{-1}), and (C) shoot above-ground (A.g.) and below-ground (B.g.) mass (g dry wt.). DB: Delvan Bay, CB: Chacalochee Bay, MP: Meaher Park, BM: Bay Minette, WH: Water Hole, TB: Turkey Branch. Values represent means ± SE (n = 40). Means sharing the same letter do not significantly differ ($P \geq 0.05$)

Meaher Park, both above- and below-ground biomass were lower in Weeks Bay (WH and TB; Fig. 2C).

3.3. Photosynthetic variables (*V. neotropicalis*)

3.3.1. Photosynthetic pigments

With the exception of Delvan Bay, chlorophyll *a* concentrations were highest in plants from Weeks Bay and lowest for plants from Bay Minette and Meaher Park (Table 3). Plants collected from the latter sites also had the lowest chlorophyll *b* and carotenoid concentrations (Table 3). Similarly, total chlorophyll concentrations were highest in Weeks Bay plants, except when compared with plants from Delvan Bay, and lowest in Bay Minette and Meaher Park plants (Table 3).

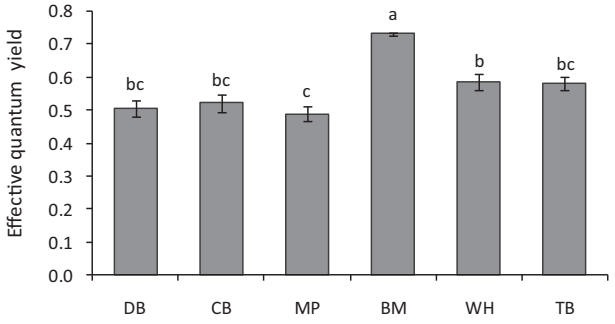

Fig. 3. *Vallisneria neotropicalis*. Effective quantum yield of photosystem II ($\Phi_{PSII} = (F_m' - F_s)/F_m' = \Delta F/F_m'$; F_m': maximum fluorescence yield in the light; F_s: steady-state fluorescence yield) per site. DB: Delvan Bay, CB: Chacalochee Bay, MP: Meaher Park, BM: Bay Minette, WH: Water Hole, TB: Turkey Branch. Values represent mean fluorescence yield ± SE; n = 80 for all sites, except DB where n = 40. Means sharing the same letter do not significantly differ ($P \geq 0.05$)

3.3.2. Chlorophyll *a* fluorescence

The highest average effective quantum yield ($\Delta F/F_m'$) was measured among *V. neotropicalis* plants growing in Bay Minette (0.731 ± 0.004; Fig. 3). Additionally, the average fluorescence yield was higher for plants growing in Water Hole than for those growing in Meaher Park (0.586 ± 0.023 *cf.* 0.489 ± 0.023; Fig. 3). No other statistically significant site differences were detected with respect to quantum yield.

3.3.3. Respiration and photosynthesis

Dark respiration rates (R_d) were significantly higher in *Vallisneria* plants growing in Water Hole (11.98 ± 2.03 μmol O_2 g fw^{-1} h^{-1}) than in Bay Minette and Chacalochee Bay (4.86 ± 0.66 and 4.23 ± 0.70 μmol O_2 g fw^{-1} h^{-1}, respectively); no other significant differences were detected among *Vallisneria* populations (Fig. 4A). The light-saturated rate of photosynthesis (P_{max}) was significantly higher in plants growing at Meaher Park relative to those growing in Chacalochee Bay (36.76 ± 1.92 *cf.* 13.19 ± 3.25 μmol O_2 g fw^{-1} h^{-1}; Fig. 4B). No other statistically significant differences were noted regarding photosynthetic rate.

3.4. As and Hg concentrations in *V. neotropicalis* leaves

As concentrations in *V. neotropicalis* leaves did not significantly vary among sites (Table 4). Although leaf As concentrations were highest in *V. neotropicalis* growing in Turkey Branch, concentrations were not statistically different from those found in populations from other sites. This result was likely attributable to substantially

Table 3
Pigment concentrations (mean ± SE; in μg g^{-1} fresh wt.) in *V. neotropicalis*.

Site	n	Chl *a*	Chl *b*	Total Chl	Carot.
DB	40	798.0 ± 25.4 ^{ab}	302.4 ± 11.4 ^{a}	1100.4 ± 36.4 ^{ab}	362.1 ± 12.0^{a}
CB	80	739.0 ± 15.0^{b}	262.7 ± 5.9^{a}	1001.7 ± 20.6^{b}	318.2 ± 7.1^{a}
MP	80	552.9 ± 12.0^{c}	184.7 ± 4.6^{b}	737.6 ± 16.4^{c}	266.1 ± 5.6^{b}
BM	80	557.9 ± 13.9^{c}	195.3 ± 5.0^{b}	753.2 ± 18.6^{c}	259.8 ± 5.9^{b}
WH	80	850.1 ± 15.3^{a}	292.5 ± 6.3^{a}	1142.6 ± 21.5^{a}	344.7 ± 5.8^{a}
TB	80	823.3 ± 12.6^{a}	290.7 ± 4.8^{a}	1114.1 ± 17.3^{a}	345.7 ± 5.5^{a}

DB: Delvan Bay, CB: Chacalochee Bay, MP: Meaher Park, BM: Bay Minette, WH: Water Hole, TB: Turkey Branch.
Chl: chlorophyll; Total Chl = Chl *a* + Chl *b*; Carot.: carotenoids.
Means sharing the same letter do not significantly differ ($P \geq 0.05$).

Table 4
As and Hg concentrations in *V. neotropicalis* leaves (mean ± SE; in μg g^{-1} dry wt. for As and in ng g^{-1} dry wt. for Hg).

	DB	CB	MP	BM	WH	TB
As (n = 3)	1.17 ± 0.03^{a}	1.90 ± 0.25^{a}	1.04 ± 0.13^{a}	2.60 ± 0.51^{a}	1.73 ± 0.09^{a}	2.77 ± 0.44^{a}
Hg (n = 9)	5.57 ± 1.10^{bc}	5.77 ± 0.55^{bc}	3.95 ± 1.04^{c}	3.76 ± 0.40^{c}	11.42 ± 1.00^{ab}	15.18 ± 1.20^{a}

DB: Delvan Bay, CB: Chacalochee Bay, MP: Meaher Park, BM: Bay Minette, WH: Water Hole, TB: Turkey Branch.
Means sharing the same letter do not significantly differ ($P \geq 0.05$).

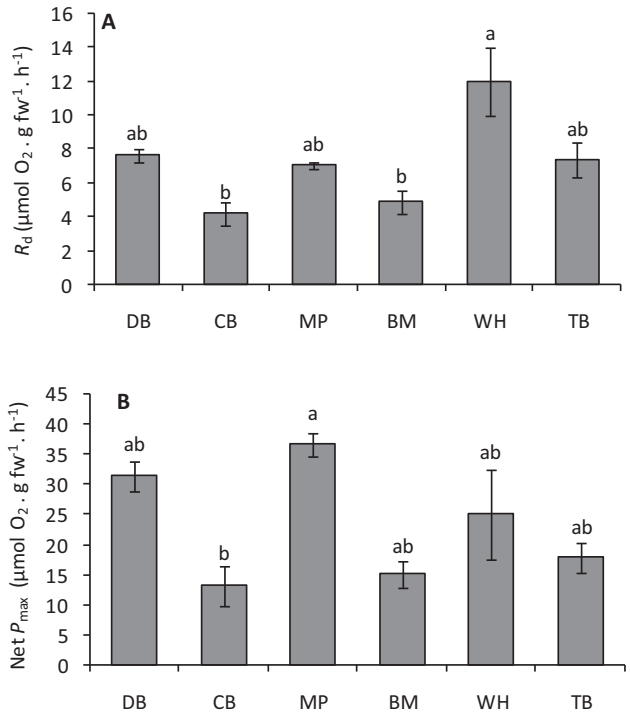

Fig. 4. *Vallisneria neotropicalis*. Laboratory gas-exchange measurements of (A) dark respiration (R_d, μmol O_2 g fw^{-1} h^{-1}) and (B) light-saturated photosynthesis at 500 μmol photons m^{-2} s^{-1} (P_{max}, μmol O_2 g fw^{-1} h^{-1}). DB: Delvan Bay, CB: Chacalochee Bay, MP: Meaher Park, BM: Bay Minette, WH: Water Hole, TB: Turkey Branch. Values represent mean ± SE (n = 4). Means sharing the same letter do not significantly differ ($P \geq 0.05$).

high within-site variability (Table 4). In contrast, leaf Hg concentrations were significantly higher in *V. neotropicalis* collected from Turkey Branch than those collected from any other site, except Water Hole. Leaf Hg concentrations were significantly higher in *V. neotropicalis* growing in Water Hole than those recorded for both the Meaher Park and Bay Minette populations (Table 4).

3.5. Associations between the morphological and photosynthetic variables measured and the As and Hg concentrations in V. neotropicalis leaves

No strong regression models were generated between morphological and physiological variables measured and leaf concentrations of As and Hg in *V. neotropicalis* (maximum value of r^2 = 0.597; Table 5). Significant regression models revealed that (i) a reduction in shoot length was correlated with high leaf Hg and low leaf As concentrations, (ii) low above- and below-ground biomass were correlated with high leaf Hg concentrations, and (iii) high chlorophyll *a* and *b* concentrations were correlated with high leaf Hg concentrations (Table 5).

4. Discussion

Plants are sensitive bioindicators that exhibit rapid and often predictable morphological and physiological responses to environmental stress. Thus, the measurement of plant characteristics along a gradient of intensity can provide important information about how stress affects overall plant performance and health. Measurements, however, can be influenced by environmental variability (other than that associated with the stressor of interest) and inter-specific competition across populations. Water-column variables (i.e., temperature, pH, DO, light attenuation, salinity, chlorophyll *a*, and nutrients) did not significantly differ among study sites (i.e., all sites except Delvan Bay for which no statistical test could be performed, n = 1). Although we observed some site-specific variation in species composition, all sites were dominated by *V. neotropicalis* and *N. guadalupensis*. These observations suggest that our site comparison and conclusions with respect to how metal/metalloid contamination affects the architecture and performance of *V. neotropicalis* are justified. Considering that few studies have addressed As levels in aquatic plants, As contamination levels among *V. neotropicalis* populations in our study area (range of mean concentrations = 1.04–2.77 μg g^{-1} dry wt.) appear to be rather low/moderate [37,79,80]. Our data do not point to a gradient in plant As contamination among sites. Similarly, Hg contamination levels in *V. neotropicalis* (range of Hg mean concentrations = 3.76–15.18 ng g^{-1} dry wt.) also appear to be

Table 5
Results for multiple regression analyses of morphological and photosynthetic variables (dependent variables) versus trace element concentrations (independent variables) in *V. neotropicalis*.

Dependent variable (DP)	Estimated regression model	r^2 (P value)
Shoot height (S.h.; cm)	S.h. = 41.13 + **14.33** As − **3.43** Hg	**0.597** (P = 0.001)
Number of leaves per shoot (No. of leaves; leaves shoot^{-1})	No. of leaves = 8.96 − 0.51 As + 0.14 Hg	0.209 (P = 0.172)
Aboveground mass (A.g.; g dry wt.)	A.g. = 0.56 + 0.14 As − **0.05** Hg	**0.558** (P = 0.002)
Belowground mass (B.g.; g dry wt.)	B.g. = 0.11 + 0.03 As − **0.01** Hg	**0.521** (P = 0.004)
Chlorophyll *a* concentration (Chl *a*; μg g^{-1} fresh wt.)	Chl *a* = 646.36 − 42.04 As + **19.98** Hg	**0.458** (P = 0.010)
Chlorophyll *b* concentration (Chl *b*; μg g^{-1} fresh wt.)	Chl *b* = 230.69 − 16.67 As + **7.21** Hg	**0.366** (P = 0.033)
Carotenoids concentration (Carot.; μg g^{-1} fresh wt.)	Carot. = 305.29 − 18.72 As + **5.98** Hg	0.314 (P = 0.059)
Effective quantum yield ($\Delta F/F_m'$)	$\Delta F/F_m'$ = 0.48 + **0.10** As − 0.01 Hg	0.315 (P = 0.059)
Dark respiration rate (R_d; in μmol O_2 g fw^{-1} h^{-1})	R_d = 7.13 − 1.28 As + **0.30** Hg	0.266 (P = 0.098)
Light-saturated photosynthesis rate (P_{max}; in μmol O_2 g fw^{-1} h^{-1})	P_{max} = 36.06 − **6.79** As − 0.02 Hg	0.282 (P = 0.083)

DP = f (As, Hg) with As and Hg representing trace element concentrations in *V. neotropicalis* leaves (in μg g^{-1} dry wt. for As and in ng g^{-1} dry wt. for Hg).
r^2: coefficient of determination.
Numbers in bold indicate a significance at the α = 0.05 level.

Table 6
Declines in the effective quantum yield ($\Delta F/F_m'$) observed following exposure to various contaminants (i.e., trace elements, petrochemicals, polycyclic aromatic hydrocarbons or PAHs) in several aquatic plant species.

Aquatic plant species	Contaminant exposure	References
Halophila ovalis	Trace elements	[31]
	Petrochemicals	[32]
	Herbicides	[33]
Lemna gibba	PAHs	[27]
Myriophyllum spicatum	PAHs	[27,28]
Zostera capricorni	Trace elements	[34,35,47]
	Petrochemicals	[36]
	Herbicides	[34,46]

low/moderate in these locales [37]. However, our Hg data point to a gradient in leaf concentration among populations, albeit a slight gradient.

Shoot growth and shoot growth-based endpoints, i.e., shoot biomass and shoot height, appear to be particularly sensitive to stress agents. Several studies have documented that xenobiotics, such as trace elements (*Z. marina*: [81,82]), polycyclic aromatic hydrocarbons (PAHs; *Lemna gibba*: [27] – *M. spicatum*: [27,28]), and herbicides (*R. maritima*: [29] – *Z. marina*: [30] – *Ceratophyllum demersum*, *V. natans*, *Elodea nuttallii*: [42]), can reduce aquatic plant growth. In this study, the highest Hg concentrations measured in *V. neotropicalis* were associated with short shoots and low overall above- and below-ground biomass, further supporting these models. Additionally, one of the populations with the lowest leaf Hg concentrations (populations measured in Bay Minette) exhibited shoots of similar height and biomass to those reported for healthy populations in south Florida [1,83]. Hence, morphometric differences across populations imply that there is a deleterious impact of Hg contamination on the growth of *V. neotropicalis*.

Chlorophyll-*a* fluorescence is a useful, non-invasive measure of energy transfer at PSII. Specifically, the *in situ* fluorescence yield ($\Delta F/F_m'$) is a proxy for the effective quantum yield of photosynthesis (i.e., overall photosynthetic performance; [84]). As PSII is generally considered to be the most damage-prone component of the photosynthetic apparatus, the first manifestation of plant stress is likely to be a decline in PSII function [84]. Numerous studies have shown that the *in situ* fluorescence yield is sensitive to xenobiotic stress in aquatic plants (e.g., [31,32,46]). Indeed, declines in the effective quantum yield ($\Delta F/F_m'$) followed exposure to various contaminants (e.g., trace elements, PAH, petrochemicals, herbicides) in several aquatic plant species (see Table 6). Thus, the effective quantum yield ($\Delta F/F_m'$) can be an early warning indicator of xenobiotic stress [27,31,36,46]. Trace elements, in particular, are known to interfere with several aspects of the photosynthetic pathway [35,85]. In light of these previous studies, our measurements of effective quantum yield indicate that the *V. neotropicalis* population at Bay Minette is the healthiest of all populations investigated in this study. The mean fluorescence yield for the *V. neotropicalis* population at Bay Minette (0.731 ± 0.004) is similar to that found in control specimens (i.e., specimens not exposed to contaminant treatments) in other ecotoxicological studies (*ca.* 0.700–0.800; e.g., *H. ovalis* [31,33], *L. gibba* [27], *M. spicatum* [27], *Z. capricorni* [35,47]). Conversely, the Meaher Park population appears to be the most stressed with respect to overall photosynthetic performance. The mean fluorescence yield for this population (0.489 ± 0.023) is similar to that found in specimens after different exposure to chemical treatment (*ca.* 0.400–0.500; e.g., *H. ovalis* after the first hour of exposure to 1 mg L^{-1} simazine treatment [33], *L. gibba* after 8 days of exposure to $\sim 10^1$ mg L^{-1} PAH treatment [27]). Values of fluorescence yield as low as 0.100–0.200 have been reported for plants subjected to acute and/or long-term chronic chemical exposure (e.g., [27,33,35]). Interestingly, the *V. neotropicalis* populations at Bay Minette and Meaher Park had the lowest leaf Hg concentrations of any reported in this study. Populations with the highest leaf Hg concentrations (Water Hole and Turkey Branch) display "intermediate" fluorescence yield values of *ca*. 0.600. These results suggest that (i) the levels of leaf Hg contamination in the populations studied do not necessarily result in photosynthetic damage or predictably affect photosynthetic performance and/or (ii) perhaps, there are other stressors present in the Meaher Park site that constrain this population's overall photosynthetic performance. Meaher Park is located adjacent to the Mobile Causeway and is therefore potentially exposed to high levels of disturbance.

Similarly, our oxygen exchange measurements do not support significant impacts of low-moderate leaf Hg contamination on rates of dark respiration and/or light-saturated photosynthesis in *V. neotropicalis* populations along the Alabama coast. The lack of significant impacts on light-saturated photosynthesis is unsurprising, given the lack of sensitivity reflected in the effective quantum yield data. The relatively higher dark respiration rates found in Water Hole and Turkey Branch relative to the other sites might represent a potential stimulation of respiratory activity and carbon loss upon exposure to moderate Hg contamination levels. In part, elevated respiratory levels might explain the overall reduction in growth and biomass exhibited by *V. neotropicalis* populations with the highest Hg leaf concentrations. However, we maintain that this is only a conjecture and warrants further investigation.

Low chlorophyll concentration and/or a reduction in chlorophyll concentration are often signs of plant stress. Declines in chlorophyll content have been frequently observed in aquatic plants following contaminant exposure [28,42,86,87]. Reductions in pigment content were suggested to primarily result from the toxic effect of contaminants on δ-aminolevulinic acid dehydratase activity, a key enzyme in the chlorophyll biosynthetic pathway [88,89]. Yet, in our study, leaf chlorophyll concentrations were highest in populations with the highest Hg leaf concentrations, and lowest in populations with the lowest Hg leaf concentrations. Several studies also noted increases in leaf carotenoid concentration following contaminant exposure [31–33,87], which is thought to be a protective mechanism against contaminant-induced oxidative stress [87,90]. On the contrary, high carotenoid content was not linked to high tissue Hg content in the present study. Thus, as is the case for the effective quantum yield, the levels of leaf Hg contamination reported here do not seem to elicit changes in pigment content in *V. neotropicalis*. It is noteworthy that several investigators have shown such deleterious impacts to mainly occur at high levels of contaminant exposure, with low contaminant dosages sometimes associated with increased chlorophyll content or having no effect [91–93].

With the exception of the overall negative effect on shoot growth and stimulatory effect on respiration, our study does not indicate that the gradient of increasing Hg contamination in leaves of *V. neotropicalis* found in northern and eastern regions of Mobile Bay is detrimental to this ecologically important plant. This is one of only a few studies that have addressed the effects of ecologically relevant levels of metal/metalloid contamination in aquatic plants. In a previous study, we reported that the study area is characterized by low to moderate sediment As and Hg contamination levels (range of As mean concentrations: 1.06–10.51 μg g^{-1} dry wt.; range of Hg mean concentrations: 12.1–72.7 ng g^{-1} dry wt.; [94]). Hence, the lack of strong, causative links between plant metal/metalloid contamination and plant responses is probably due to low-level exposure. However, this is not to say that low-level chronic exposure has no effect over the long-term. Most concentrations applied in laboratory experiments involving metal/metalloid toxicity are extremely high and rarely found in nature. Graney et al. [44] questioned the applicability of laboratory predictions to the real world because chemicals are often less (but sometimes more) toxic in a

complex environment. Moreover, aquatic plants, especially those such as *V. neotropicalis* that typically inhabit highly variable estuarine habitats, possess physiological mechanisms to cope with environmental stress. These mechanisms include those induced by metal/metalloid contamination in an effort to minimize impact(s) (e.g., mechanisms of metals/metalloids detoxification and tolerance [95–98]). Finally, unanticipated environmental differences might have masked the impacts of the metal/metalloid contamination gradient across study sites and populations.

Despite the fact that we found no conclusive evidence to support our supposition that metal/metalloid contamination has significant effects on the structure and function of *V. neotropicalis* in the field, we maintain that these data are important for ecological impact assessment purposes. First, herein we provide novel information concerning ambient metal/metalloid concentrations in *V. neotropicalis* tissues that might be useful for future studies designed to address the biology and ecology of aquatic plants, and their potential use as biomonitors. Moreover, given the pivotal role of plants as providers of food and shelter for a myriad of herbivores and detritivores, our results represent important baseline data for assessments of metal/metalloid bioamplification through trophic webs in shallow coastal ecosystems (i.e., systems that are often subjected to high anthropogenic pressure). Second, our results suggest that the aquatic plant, *V. neotropicalis*, has the ability to tolerate moderate levels of metal/metalloid contamination with little impact on its performance. Third, these data highlight the importance of taking both whole-organism as well as cellular responses into consideration when addressing ecotoxicological questions because environmental effects are often disparate. Such "multi-level" approaches have come to the forefront in the field of ecotoxicology. Our data suggest that an evaluation of tissue contaminant concentrations alone is insufficient to determine the ecological risks associated with pollution of aquatic systems. Rather, a combination of chemical and biological assessments is necessary to establish links between contaminant levels and potential toxicological impacts. Clearly, more studies are needed (i) to assess and understand the long-term impacts of chronic exposure to low/moderate metal/metalloid environmental contamination in natural populations of aquatic plants, (ii) to better understand aquatic plant responses and tolerance mechanisms (e.g., scavenging and detoxification) as they relate to metal/metalloid contamination, and (iii) to evaluate the potential of various morphological and physiological parameters as effective early biomarkers of environmental disturbance associated with metal/metalloid contamination in aquatic systems. Our study represents an initial step toward achieving these goals.

Acknowledgments

This research was supported by a grant from the Alabama Center for Estuarine Studies (**R83-0651), a Center supported by the U.S. Environmental Protection Agency, and a grant from the "Collectivité Territoriale de Corse". The authors thank Dr. S. Phipps and E. Brunden from the Weeks Bay NERR for their assistance in the field. Additionally, we wish to thank S. Glenos, K. VanDeven, B. Christiaen, J. Goff, L. Marino, L. Moore, and A. McDonald for support in both field and laboratory settings. We also thank the editor of the *Journal of Hazardous Materials* and two anonymous reviewers for their helpful comments and suggestions that greatly improved this manuscript.

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