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Posidonia oceanica and *Zostera marina* as Potential Biomarkers of Heavy Metal Contamination in Coastal Systems

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1. Introduction

In the early 1960s recognition of the adverse effects of environmental contamination due to industrial, pesticide, and agricultural pollution led to the emergence of the field of ecotoxicology (Ramade, 1992). Today, marine estuary and inshore ecosystems continue to be negatively impacted by environmental contamination (Short & Wyllie-Echeverria 1996; Orth et al., 2006; Osborn & Datta, 2006). In order to reduce these negative impacts, bio-surveillance programs are needed to monitor environmental conditions so that changes in ecosystem processes, structure, and the physiological condition of species can be assessed (Blandin, 1986; Tett et al., 2007). An important characteristic of these programs is that indicator species must be capable of rapidly detecting significant changes in the ecosystem so that the cause of deterioration can be addressed early (e.g. Hemminga & Duarte, 2000).

Mussels (Goldberg et al., 1983) and fish (Reichert et al., 1998; Stephensen et al., 2000) are frequently used as indicators of chemical contamination in long-term environmental monitoring programs. However, these programs can be deficient because they only provide information about water column contamination, and these organisms can have limited ranges and often must be introduced to a site as part of the monitoring program. To offset these deficiencies widely distributed indicator organisms in coastal systems that have the capacity to provide contamination information from both water column and sediment environments are needed. Consequently, there is increasing interest in the use of marine macrophytes because they grow in most coastal and estuarine systems (see Green & Short, 2003). These rooted vascular plants interact with the chemical properties of the water column and surface sediment environments within site-specific and basin-wide locations (Brix, 1997; Brix et al., 1983; den Hartog, 1970; Lange and Lambert, 1994; Rainbow and

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Phillips, 1993). For this study the focus was on the seagrasses *Posidonia oceanica* (L.) Delile (Posidoniaceae) and *Zostera marina* L. (Zosteraceae). These were chosen because they are the dominant species in the regions of our inquiry which were, respectively, the Mediterranean Sea (Lipkin et al., 2003; Procaccini et al., 2003) and the Pacific Northwest (Wyllie-Echeverria & Ackerman, 2003). Both species can form vast meadows across the intertidal-subtidal gradient in their respective ecosystems (Molinier & Picard, 1952; Phillips, 1984).

1.1 *P. oceanica* and *Z. marina* as indicators of environmental quality

The potential for these species to provide an early warning of deteriorating environmental quality has been noted for *P. oceanica* and *Z. marina* where both species were found useful at detecting environmental deterioration within local and basin-wide locations (Augier, 1985; Dennison et al., 1993; Pergent, 1991; Pergent-Martini et al., 1999). For example, *P. oceanica* accumulates certain metal pollutants, notably mercury (Pergent-Martini, 1998), which is one of the most abundant marine pollutants. Within the Mediterranean Sea elevated mercury levels have been reported in certain regions (Maserti et al., 1991), and correlations have been drawn between mercury levels in plant tissue and the concentrations of mercury in the water column (Pergent-Martini, 1998). In laboratory studies Lyngby & Brix (1984) and Brix & Lyngby (1984) demonstrated that *Z. marina* can accumulate heavy metals in concentrations above natural levels, and that these concentrations inhibited growth. In addition, based on extensive sampling along the coastline of Limfjord, Denmark, these authors noted that *Z. marina* could be used to monitor heavy metal contamination. Also, a related species *Z. capricorni* has provided valuable information in monitoring iron, aluminium, zinc, chromium, and copper contamination (Prange & Dennison, 2000).

Indicator species that provide an early warning of ecosystem change will likely be those that reveal first order changes in organism function. Molecular, biochemical, and/or cellular changes triggered by pollutants are measurable in biological mediums such as cells, tissues, and/or cellular fluids (McCarthy & Shugart, 1990). For example, oxidation is known to be a significant factor in stress-related organismal weakening, and antioxidant molecules have been used to evaluate organism health (Chen et al., 2007). One group of antioxidant molecules are the widely studied phenolic compounds (Ferrat et al., 2003a) which are known to be induced by reactive oxygen species (Rice-Evans et al., 1995; Vangronsveld et al., 1997).

1.2 Physiological and ecological roles of phenolics and volatile compounds

Phenolic compounds produced via the Shikimic Acid Pathway, and volatiles produced via the Mevalonate Pathway, are known to be important to plant health and survival (Cates, 1996; Fierer et al., 2001; Hartman, 2007; Phillips, 1992; Schimel et al., 1996). They are found in terrestrial higher plants, most notably angiosperms (Goodwin & Mercer, 1983; Hadacek, 2002), some seagrasses (Verges et al., 2007; Zapata and McMillan, 1979), and have a wide range of chemical structures and activities (Hadacek, 2002; Hartman, 2007). Phenolic and volatile compounds contribute significantly to the antioxidant activity of plants, have the capacity to bind heavy metals (Emmons et al., 1999), and are an important mechanism in protecting plants against stress (Swain, 1977). Volatile compounds (e.g. monoterpenes, sesquiterpenes) have been found to serve as energy sources in plants (Croteau & Sood,

1985), are important in the defensive system of higher plants (Cates, 1996; Langenheim, 1994), and influence ecosystem processes such as nutrient cycling (Horner et al., 1988; White, 1986). The production of phenolics and volatiles is under genetic control (Croteau & Gershenzon, 1994; Hartman, 2007), but their qualitative and quantitative production is affected by various environmental factors (Bryant et al., 1983; Gershenzon, 1984; Hartman, 2007; Macheix, 1996; Quackenbush et al., 1986; Ragan & Glombitza, 1986). However, as with other seagrasses, only a very limited number of studies deal with the role of phenolic and volatile compounds from *Posidonia oceanica* (Heglmeier & Zidorn, 2010) and *Zostera marina* (Short & Willie Echeverria, 1996). Only were investigated the impacts of interspecific competition (Dumay et al., 2004), nutrient variation, diseases (Vergeer & Develi, 1997) and grazing (Cannac et al., 2006), or general anthropization of water masses (Short & Wyllie-Echeverria, 1996, Agostini et al., 1998).

1.3 Objective

The objective of this study was to determine if *P. oceanica* and *Z. marina* might be reliable candidates as bio-surveillance organisms with regard to heavy metal pollution. We choose to consider different environmental conditions and to monitor physiological changes through two different seasons. Our assumption was that heavy metal contamination would adversely impact adult *P. oceanica* and *Z. marina* plants, and that plant response to these impacts could be assessed by differences in phenolic and volatile compound content of tissue from impacted and non-impacted sites.

We assessed differences in heavy metal content of plant tissues from sites with documented heavy metal pollution versus controls with no sources of heavy metal pollution. Then, we tested the hypothesis that the presence of identified contaminants could induce a bio-indicator response in these seagrass species. To do this we measured changes in total phenolic content in the leaf and sheath tissue of *P. oceanica*, and total phenolic and volatile compound content in above-and below-ground tissue of *Z. marina*.

2. Materials and methods

2.1 Site location and sample collection

In June 2000 and January 2001, 30 adult shoots of *P. oceanica* were collected by SCUBA at ~10 m in the sub-tidal region at two sites located in the northwestern Mediterranean Sea. The Bay of Bonifacio, a control site, is a pristine area relatively free of industrial pollution located in the south of Corsica (Tonnara - France; 41.4000 N; 9.0830 E; Capiomont et al., 2000). The Bay of Rosignano site south of Livorno (Italy; 43.4000 N; 10.4166 E) is a polluted site. At this site, a chlor-alkali plant has discharged industrial wastes rich in mercury since 1920 (130 kg per year; Ferrara et al., 1989). Water temperature ranged from 18°C in June 2000 to 14°C in January 2001 at all sites but salinity was relatively constant at 38.5 PSU within the study zone (i.e., 10 m depth contour; Villefranche sur Mer Observatory and Di Martino, personal communication).

For *P. oceanica*, foliage leaf and sheath tissue was analyzed for mercury and phenolic content. Tissue was obtained by separating the foliage leaf and sheath tissue from the roots and rhizomes following the procedure of Giraud (1977); root and rhizome tissue was discarded. The chlorophyllous foliage leaves were then separated from non-chlorophyllous

sheaths that are located at the leaf base. Foliage leaves from three adult shoots were dissected according to Giraud (1977) and combined to form one sample. Sheaths from the same three shoots were combined to form each sheath sample. After epiphytes were removed from leaf and sheath samples using a glass slide, each sample was rinsed with ultra-pure water and frozen (-20°C) until analysis. To determine mercury and phenolic content, we extracted 0.5 g dry wt. of each tissue sample (n=10).

During maximum low tide, *Z. marina* adult shoots were hand-collected from the lower intertidal region of two sites in Northern Puget Sound, Washington, USA in April and June 2000. The site located near Anacortes, WA (48.4263 N; 122.5897 W) was documented as having heavy metal pollution (http://www.ecy.wa.gov/programs/wq/permits/permit_pdfs/dakota/factsheet.pdf), and the other was a pristine location with no industrial activity on the southeast side of Shaw Island (48.33942 N; 122.55448 W) that served as the control site (Wyllie-Echeverria & Ackerman, 2003). Water temperature ranged between 9°C in April to12°C in June 2000 at all sites, and salinity was relatively constant at 30 PSU during this time period (Wyllie-Echeverria, unpublished data).

Three samples were collected from each site, and each sample consisted of at least 0.5 g dry wt (Cuny et al., 1995) of eight to ten sterile (non-reproductive) shoots which were separated into above- and below-ground parts. Above-ground tissue consisted of the foliage leaf (i.e. basal leaf sheath and distal leaf blade; Kuo & den Hartog, 2006) excised from the rhizome at the node primordia (Tomlinson, 1974). The remaining rhizome and associated nodes and roots formed the below-ground sample. Epiphytes were scraped from the above-ground tissue and sediment was rinsed from the roots and rhizomes (Brackup & Capone, 1985). Each above- and below-ground sample was placed in labelled bags, kept moist and cool in a refrigerator, and shipped overnight to the Chemical Ecology Laboratory at Brigham Young University. Three replicate samples of above-ground tissue from each site, and three replicates of below-ground tissue from each site, were frozen at –80°C until extracted for heavy metals, phenolics or volatiles. Samples were stored at –80°C to preserve the volatile compounds in the tissues.

2.2 Qualitative and quantitative analysis of plant tissues for heavy metal content

Foliage leaves and sheaths of *P. oceanica* and above- and below-ground tissues of *Z. marina* were analyzed qualitatively and quantitatively for heavy metals. For *P. oceanica*, only mercury content, which is the predominant heavy metal pollutant at the Rosignano site (Lafabrie et al., 2007), was analyzed. Three individual shoots (three foliage leaves and three sheaths) that had been separately freeze dried were ground to a powder, and an aliquot of 0.05 g dry wt was digested. Digestion was performed in a 100-ml Teflon® advanced composite vessel reactor with 5 ml HNO_3 and 1 ml H_2O_2 (30%). Microwave digestion (Mars 5, CEM Chemistry, Engineering and Microwave, Matthews, NC, USA) was carried out using a temperature ramp of 8 min up to 200°C followed by a heating plateau of 20 min at 200°C. After digestion, the samples were increased to 25 ml with ultra-pure water and then filtered. Total mercury was determined using a flameless atomic absorption spectrophotometer flow injection (Perkin-Elmer System 100; Norwalk, CT, USA). The procedure consisted of reduction with 1.1% tin chloride ($SnCl_2$, $2H_2O$) in 3% HCl and 0.5% hydroxylammonium chloride (NH_2OH, HCl). A standard addition method for total mercury was used to calibrate the protocol. The analytic procedure was verified using a moss as the certified

reference material (*Lagarosiphon major*, Certified Reference Material 60, Community Bureau of Reference, Commission of the European Community, Brussels, Belgium). Data are expressed as ng per g dry wt.

For *Z. marina*, heavy metal content was analyzed using the EPA Method 3052 Procedure. All elements were wet-ashed to prevent loss of elements and reduce the potential of confounding data due to silica content. Above- and below-ground tissue (0.5 g dry wt) was placed in a 50 ml folin tube, and 5 ml concentrated nitric acid was added. Samples sat overnight, and then were placed on a block digester at 200°C for 5-10 minutes. Tubes were removed, cooled, and then digested with 1 ml hydrofluoric acid. Samples were placed back on the block digester for 45-60 minutes. Tubes were removed and brought to a 50 ml volume with distilled water. Stoppered tubes were shaken and then analyzed by inductively coupled plasma atomic emission spectrometry (Iris Intrepid II XSP, model 14463001; Thermo Electron Corporation, Franklin, MA) equipped with an ASX-520 autosampler. Data are expressed as ppm (Table 2).

2.3 Extraction and determination of phenolic content in the tissues of *P. oceanica*, and phenolic and volatile content of above-ground tissues of *Z. marina*

Total phenolic content for both species, and total volatile content for *Z. marina*, were determined to ascertain whether tissue collected from impacted (heavy metal pollution for both species) and control sites differed. A different method is used for the definition of the phenolic and volatile compounds, because the measurements were realized in different labs. For *P. oceanica*, extraction of total phenolic compounds was carried out on 0.5 g dry wt freeze-dried foliage leaf or sheath tissue. Extraction followed Cuny et al. (1995) and consisted of infusing each sample at 40°C in 50 % (v/v) aqueous ethanol in darkness for 3 h. The extract was acidified with a few drops of 2N HCl, the ethanol was evaporated under vacuum, and the aqueous residue extracted with ethanol/acetic acid. The organic phase was dried using anhydrous Na_2SO_4. Concentration of total phenolic compounds was measured by colorimetry (Swain & Hillis, 1959) using Folin-Denis reagent (Folin and Dennis, 1915). Phloroglucinol (Frantzis, 1992) was used for elaboration of standard curves. For *Z. marina*, phenolics were extracted using MeOH/CH_2CH_2 (50/50) from 200 mg dry wt of freeze dried above-ground tissue, filtered using VWR grade 415 filter paper, and blown dry using nitrogen gas to prevent oxidation. After resdisolving in MeOH/CH_2CH_2 (50/50), the extract was again filtered, placed in an auto-sampler vial (Chromatography Research Supplies, Addison, IL) and injected into a high pressure liquid chromatograph (HPLC) (HP Model 1100; Agilent 1100 Series, Model G1313A; Santa Clara, CA) equipped with a diode-array detector (Model G1316A) and a C_{18} reverse phase 5µm column (Phenomenex, Torrance, CA). The HPLC solvents were A = water/acetic acid (98:2); B = acetonitrile/acetic acid (98:2). Temperature was 50°C, flow rate 1ml/min, and wavelength of the detector set at 280 nm (optimized for *Z. marina* phenolic compounds). Phenolic content is expressed as total peak height /200 mg dry wt. To obtain volatile compound content in *Z. marina* samples, 3 g fresh wt of above-ground tissue was ground to a fine powder in liquid nitrogen and hexane. The extract was then filtered, and the filtrate injected into a capillary gas chromatograph (HP Model 6890) equipped with a head-space sampler (Perkin-Elmer HS 40 XL; Waltham, MA) and a HP-1 column. Oven temperature was 80°C, needle temperature 85°C, transfer temperature 120°C, thermostat time 10 min, pressurizing time 0.6 min, injection time 0.2

min, and withdrawal time 0.5 min. The ramp GC program was 40-210°C at intervals of 3°C ramp/min. Total volatile compound content is expressed as total peak height per 3 g fresh wt tissue.

2.4 Statistical analysis

Data from *P. oceanica* samples were analyzed using a three-way ANOVA to allow comparisons between the phenolic compounds and mercury levels according to tissue, site and sampling period. Since the interaction among these factors was significant, one-way analyses followed by a Tukey test (for analyses over the annual cycle) or Student-t test (for analyses of tissue and site factors at given months) were performed (Zar, 1999). Normality and homoscedasticity were verified by Shapiro Wilks and Bartlett tests, respectively (Zar, 1999). The relationships between phenolic compounds and mercury level were assessed using correlation and regression analyses in Statgraphics plus (ver 3.1) for Windows. Data from *Z. marina* are expressed as ppm for heavy metals, total peak area per 200 mg freeze dried tissue for phenolics, and total peak area per 3 g fresh wt for volatiles. Since all samples were randomly collected along a transect, each sample is treated as an independent experimental unit. Comparison of heavy metal content between impacted and control sites in *Z. marina* above- and below-ground tissues, and for phenolic and volatile content in above-ground tissues, was conducted using a one-way ANOVA, SAS GLM program (SAS, 1996).

3. Results

3.1 Site and tissue differences in heavy metal contamination

Foliage leaf and sheath tissue of *P. oceanica* from the industrially impacted Rosignano site showed large and significant ($p<0.05$) differences in mercury content when compared to the control Tonnara site (Table 1).

Tissue Type		Mercury impacted site (Rosignano)	Control site (Tonnara)
Foliage Leaves	June 2000	233 ± 23	77 ± 11
	January 2001	317 ± 41	79 ± 15
Sheaths	June 2000	368 ± 26	64 ± 8
	January 2001	215 ± 16	80 ± 19

Table 1. Mercury levels (ng/g dw) in foliage leaf and sheath tissues of *P. oceanica* collected at different sites and different sampling periods.

Samples of above-ground tissue collected in April 2000 from *Z. marina* plants growing in the impacted site were higher in iron, aluminium, and copper when compared to tissue from the control site (Table 2). However, above-ground tissue from the control site was significantly higher in zinc, nickel, molybdenum, and mercury when compared to the impacted site (Table 2). For the July 2000 samples, the only significant differences were that nickel and copper were in highest concentration in plants from the impacted site when compared to plants from control site (Table 2). For below-ground tissue of *Z. marina* in April, samples from the industrially impacted site were significantly higher ($p<0.05$) for iron, aluminium, nickel, manganese, copper, cadmium, chromium, and lead when

compared to the control site (Table 2). None of the heavy metals was higher in concentration in the control site for samples taken in April 2000. For the July 2000 samples, barium, iron, aluminium, zinc, manganese, copper, cadmium, arsenic, and chromium were higher in the plants from the impacted site when compared to the control site, and cobalt and strontium were higher in plants from the control site (Table 2).

Heavy Metals	Site (ppm)*							
	Above-ground Tissue				Below-ground Tissue			
	April		July		April		July	
	Industrially impacted site	Control site	Industrially impacted site	Control site	Industrially impacted site	Control site	Industrially impacted site	Control site
Barium	323(41)^a	364(32)^a	279(107)^a	312(55)^a	466(136)^a	420(100)^a	570(148)^a	315(84)^b
Iron	320(127)^a	180(58)^b	204(87)^a	142(94)^a	5801(2846)^a	1068(540)^b	5591(1503)^a	576(263)^b
Aluminum	183(75)^a	119(43)^b	88(42)^a	100(81)^a	1626(1341)^a	665(435)^b	1737(494)^a	503(336)^b
Zinc	100(11)^a	119(13)^b	102(22)^a	110(15)^a	134(45)^a	133(44)^a	169(46)^a	96(16)^b
Nickel	55(21)^a	104(25)^b	45(16)^a	23(15)^b	127(78)^a	34(13)^b	63(20)^a	64(31)^a
Manganese	37(6)^a	42(6)^a	48(16)^a	51(7)^a	38(33)^a	11(6)^b	26(7)^a	10(4)^b
Copper	14(2)^a	12(2)^b	16(4)^a	10(1)^b	40(21)^a	19(29)^b	43(12)^a	10(3)^b
Molybdenum	5(2)^a	6(1)^b	7(2)^a	8(1)^a	0(0)	----**	0(0)^a	0(1)^a
Cadmium	2(1)^a	1(1)^a	2(1)^a	2(1)^a	13(8)^a	4(2)^b	11(3)^a	3(1)^b
Arsenic	4(2)^a	3(2)^a	3(2)^a	3(2)^a	8(7)^a	8(1)^a	10(6)^a	1(2)^b
Cobalt	2(1)^a	4(1)^a	2(1)^a	2(1)^a	2(1)^a	1(1)^a	1(1)^a	2(1)^b
Mercury	1(1)^a	2(1)^b	0(1)^a	1(1)^a	3(3)^a	2(1)^a	4(1)^a	4(2)^a
Strontium	1(2)^a	2(3)^a	4(3)^a	4(2)^a	3(4)^a	6(4)^a	0(0)^a	1(2)^b
Chromium	1(1)^a	2(1)^a	1(0)^a	1(0)^a	7(4)^a	2(1)^b	6(2)^a	1(1)^b
Lead	0(0)^a	1(2)^a	0(0)^a	0(0)^a	13(23)^a	6(3)^b	0(1)^a	0(1)^a

Table 2. Differences in accumulation of heavy metals in above- and below- ground tissues of *Z. marina* between impacted and control sites [April, July 2000; x, -]. *Means followed by different letters are significantly different at $p < 0.05$; Means followed by the same letter (i.e. "a") are not significantly different at $p < 0.05$. **Insufficient sample for analysis.

3.2 Production of phenolic and volatile compound content in plant tissues between impacted and control sites

Foliage leaves from Tonnara (20.5 mg.g $^{-1}$) were significantly higher (Tukey test, $p< 0.05$) in phenolic content in January 2001 compared to plants from the mercury impacted Rosignano site (13.2 mg.g^{-1}), but were not significantly different in the June 2000 samples (Fig. 1). For sheaths, the levels of total phenolic compounds from Tonnara plants in June and January (9.2 and 15.2 mg.g^{-1}, respectively) were significantly higher than those measured in plants at

the Rosignano site (5.0 and 6.4 mg.g^{-1}, respectively) (Tukey test, $p<0.05$). Phenolic content was higher across sites and sampling times in *P. oceanica* foliage leaves compared to sheaths in all comparisons (Mann and Whitney test, $p> 0.05$).

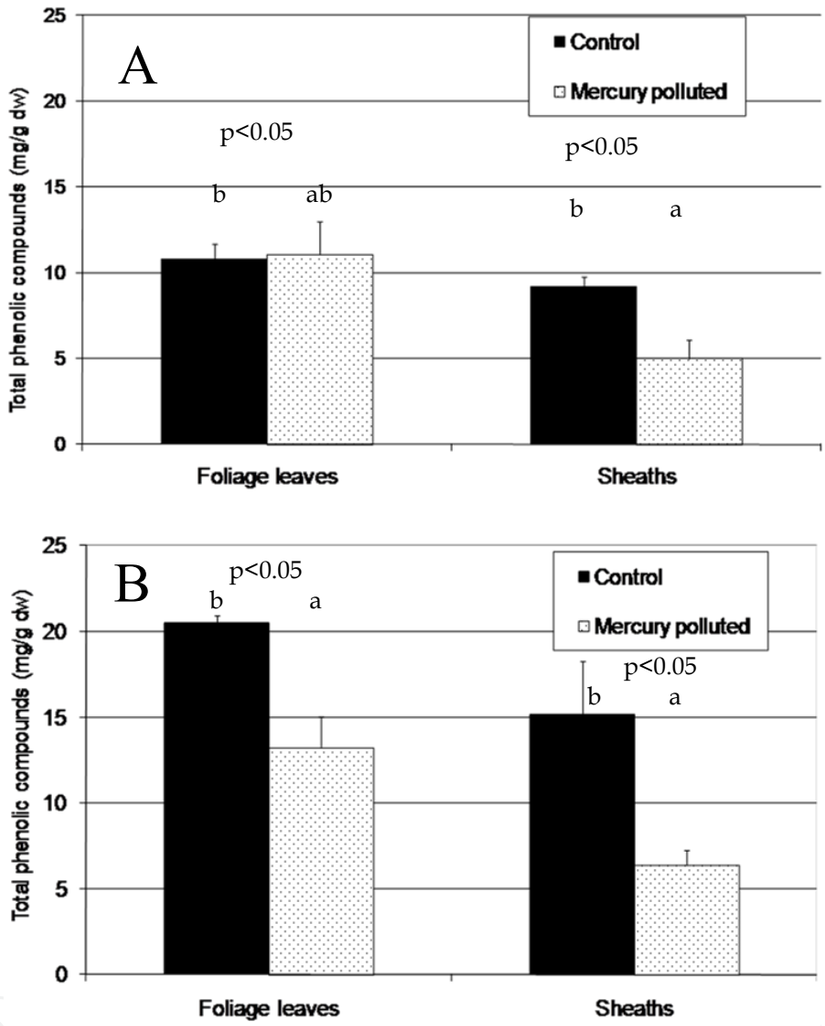

Fig. 1. Total phenolic concentration (mg.g^{-1} dw) in foliage leaf and sheath tissues of *P. oceanica* in Tonnara (control) and Rosignano (mercury polluted) in June 2000 (A) and January 2001 (B).

For *Z. marina* total phenolic content in above-ground tissues collected from plants at the control site always was higher when compared to above-ground tissues collected from the impacted site for both April and July 2000 (Fig. 2). However, the only significant difference was in July where the control site produced a higher amount of total phenolic (65.8 vs 50.8 peak area / 200 mg dry wt, respectively; $p<0.05$). Total volatile compound production also was higher at both sampling periods, but the only significant difference occurred in the April 2000 sampling where above-ground tissues from the control site showed an average peak area of 551 per 200 mg dry wt tissue compared to 352 at the impacted site ($p<0.05$).

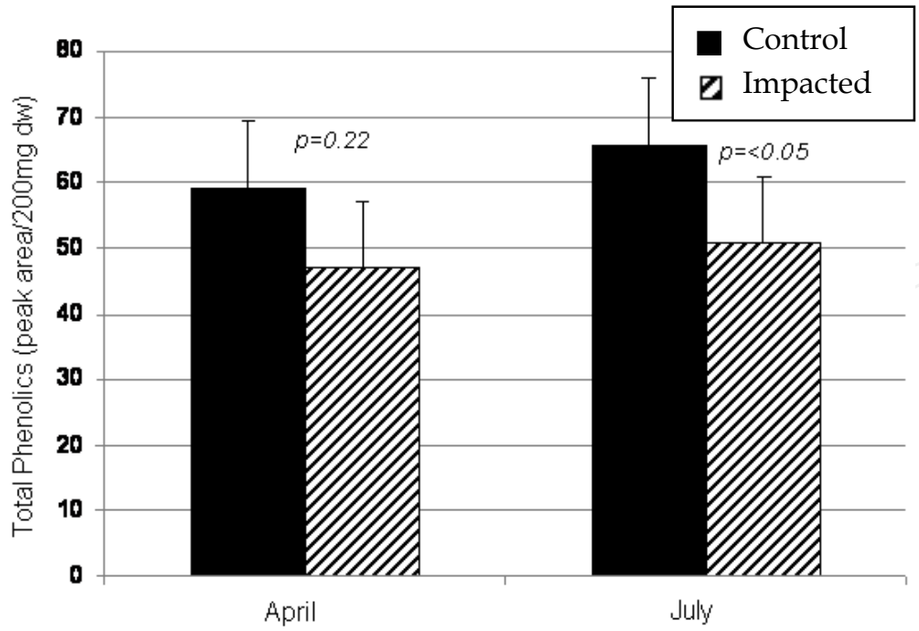

Fig. 2. Total phenolic content in above-ground tissue from *Z. marina* plants growing in heavy metal impacted and control sites (April and July, 2000).

4. Discussion

4.1 Tissue and site differences in heavy metal content

Results presented indicate that plant tissues of *P. oceanica* and *Z. marina* significantly accumulated high levels of heavy metals when growing on heavy metal-impacted sites (Tables 1 & 2). At the Rosignano site, when compared to the control Tonnara site, foliage leaves and sheaths contained two to over six times the amount of mercury. These patterns of accumulation are consistent with findings by other authors who have studied the same sites (Capiomont et al., 2000; Ferrat, 2001; Ferrat et al., 2003b; Maserti & Ferrara, 1991).

Z. marina plants from the heavy-metal impacted site accumulated significantly higher concentrations of iron, aluminum, nickel, and copper in their above-ground tissues when compared to the control site (Table 2). In addition, below-ground tissue of *Z. marina* plants from the industrially-impacted site accumulated over three, and up to five, times the levels of heavy metals compared to plants from the control site. A striking difference between above- and below-ground tissue, is that below-ground tissue from the impacted site accumulated 12 heavy metals (barium, iron, aluminum, zinc, nickel, manganese, copper, cadmium, arsenic, cobalt, chromium, lead; Table 2) while above-ground tissue only accumulated four heavy metals (iron, aluminum, nickel, copper) (Table 2). Another major difference is that the quantity of heavy metals accumulated in the below-ground tissue was higher for most of the heavy metals compared to that in the above-ground tissue.

Variation in metallic accumulation between above- and below-ground seagrass tissue has been discussed by various authors (see synthesis in Pergent Martini & Pergent, 2000), and could be a function of differences in binding sites or seasonal translocation between above- and below-ground structures (Libes & Boudouresque, 1987; Ward, 1987). The level of

environmental contamination within a particular site also may be an important factor. For example Capiomont et al. (2000) found that mercury content was higher in the interstitial water than in the water column at our Rosignano sampling location.

Heavy metals are known to have adverse affects on the physiology of *P. oceanica* and *Z. marina* as well as other seagrasses (Ward, 1987). Lyngby & Brix (1984) have shown that the order of heavy metal inhibition of growth of *Z. marina* from greatest to least is mercury, copper, cadmium, zinc, chromium, and lead. Interestingly mercury was not significantly accumulated by *Z. marina* at our impacted site but the other five generally followed the pattern described by Lynby & Brix (1984) (Table 2).

4.2 Phenolic and volatile compound production in plant tissues between impacted and control sites

Our results suggest that total phenolic compound levels within seagrass tissue could be an indicator of site quality. Differences in production of phenolics in tissues from both species were noted between impacted and control sites. For foliage leaves and sheaths of *P. oceanica* collected in January, and above-ground tissue of *Z. marina* collected in July, total phenolic content was significantly lower in plants collected from industrial sites (Fig. 1 & 2). This is supported by Vergeer et al. (1995) who concluded that a decrease of total phenolic compounds in the tissue of *Z. marina* indicated plants may be growing in unsuitable environmental conditions. Noteworthy is that correlation analysis indicated a significant ($p < 0.05$) inverse relationship between heavy metal content and the health of plants as measured by phenolic content for *P. oceanica* ($r^2 = 69.8$ %, linear model of regression: mercury = 0.22 – 0.0055 * phenol for sheaths).

Additionally, gas chromatography analysis of volatile compounds from *Z. marina* indicated that above-ground tissue from plants growing in the impacted site was significantly lower in volatiles from the April collection, when growth begins in Northern Puget Sound (Phillips, 1984) compared to tissue from the control site (Fig. 3). However, no significant differences occurred in volatile compound production between impacted and control sites in the July collection.

4.3 Phenolic compound production with regard to tissue and time collection

For *P. oceanica*, the concentration of phenolic compounds differed between foliage leaves and sheaths being higher in leaf tissue regardless of site. Similarly, Agostini et al. (1998) found higher concentrations (6 $mg.g^{-1}$) in the apical parts and youngest leaves and lower concentrations in sheaths (0.1 $mg.g^{-1}$). Also, in our study significant variation was observed between seasons; for example, phenolic levels were found to be higher in the January 2001 samples compared to the June 2000 samples.

Differences occur in the natural products analyzed depending on month of collection for both *P. oceanica* and *Z. marina (Fig. 1-3)*. For example, *P. oceanica* foliage leaves and sheaths in January 2001 were higher in phenolic content than those collected in June 2000 (Fig. 1). While phenolic content in above-ground *Z. marina* tissue was similar in concentration between April and July (Fig. 2), but volatile compounds in above-ground tissue collected in April were significantly higher than those collected in July (Fig. 3). April and July were selected as sampling times for *Z. marina* because they represent early and mature tissue

growth in the Northern Puget Sound (Phillips, 1984). However, in a preliminary study in which *Z. marina* shoots were collected in February 2000, plants from the heavy metal-impacted site produced only 19% of the total phenolic content when compared to plants from the control site (Zou et al., unpublished data). In order to establish when phenolics and volatiles may best indicate plant health, experimental designs need to involve sampling plants every two months throughout the year.

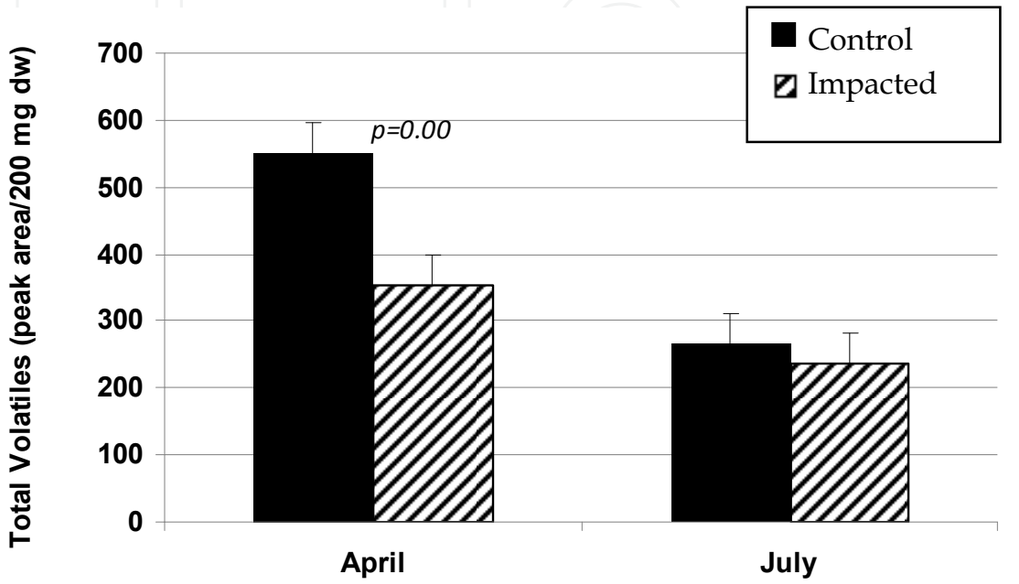

Fig. 3. Total volatile content of above-ground tissue from *Z. marina* plants growing in heavy metal impacted and control sites (April and July, 2000).

Finally, based on the response of different seagrass genotypes to disturbance (e.g. Ehlers et al., 2008; Hughes & Stachowicz, 2009; Wyllie-Echeverria et al., 2010), we suspect that variation in the type and concentration of heavy metal uptake may exist within different genotypes. However, this aspect of heavy metal accumulation in needs investigation in controlled conditions with seagrass species from different locations.

5. Conclusions

Significant differences were found in the accumulation of mercury in leaf and sheath tissues of *P. oceanica* when plants were growing on impacted sites as compared to sites not impacted heavily by mercury (Table 1). *Z. marina* plants growing in a site impacted by heavy metals associated with industrial pollution accumulated significantly higher amounts of iron, aluminium, nickel, and copper in above-ground tissues as compared to a non-impacted site, and higher amounts of barium, iron, aluminium, zinc, nickel, manganese, copper, cadmium, arsenic, chromium, and lead in below-ground tissues at the impacted site (Table 2). For *P. oceanica,* total phenolics were significantly higher in leaves at the control site when compared to the mercury impacted site for the January sampling period (Fig. 1). For sheath tissue total phenolics from the control site were significantly higher when compared to the mercury impacted site for both sampling periods (Fig. 1). For *Z. marina*, total phenolic content was higher in both sampling periods at the non-impacted site compared to the

control site, but only significantly so for the July 2000 sampling period (Fig. 2). Total volatile content also was higher at the control site for both sampling periods, but only significantly higher for the April sampling period (Fig. 3). These results support the hypotheses that *P. oceanica* and *Z. marina* accumulate significant amounts of heavy metals from impacted sites, and that these accumulations are associated with reduced total phenolic and volatile compound content. Based on these supportive data, we conclude that *P. oceanica* and *Z. marina* are potential candidates as bio-surveillance organisms especially with regard to heavy metal pollution of coastal and estuarine ecosystems.

Since we observed variation in the production of phenolics and volatiles with regard to sampling time and season, a priority is the identification of individual phenolic and volatile compounds in the tissue of these two species. In our labs we have identified in one or both species using gas chromatography/mass spectroscopy and high pressure liquid chromatography several cinnamic acid and benzoic acid derivatives; these results are comparable to those found by Quackenbush et al. (1986). Additionally, these analyses indicate not only a quantitative decrease in total phenolic and volatile compounds, but also qualitative differences between plants growing on impacted and non-impacted sites (Ferrat et al., unpublished data for *P. oceanica*; Zou et al., unpublished data for *Z. marina*). Finally, since various environmental perturbations may adversely affect seagrass health (impact of human activity reviewed in Short & Wyllie-Echeverria, 1996), and thereby phenolic and volatile compound production, collaboration among scientists working at a diversity of sites would greatly facilitate progress toward this bio-surveillance effort.

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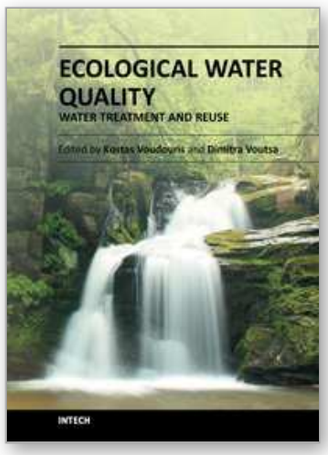

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