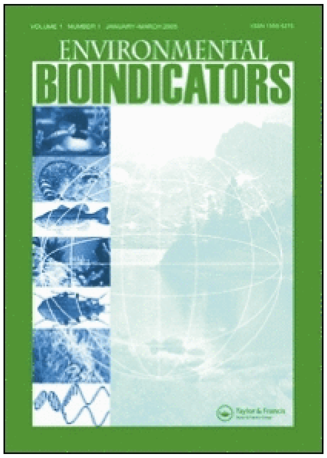

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Translational Fluctuating Asymmetry and Leaf Dimension in Seagrass, *Zostera capricorni* Aschers in a Gradient of Heavy Metals

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Methodology for measuring translational fluctuating asymmetry (TFA) on leaves of seagrass, Zostera capricorni *Aschers has been developed and tested to detect a subtle effect of environmental stress associated with heavy metal pollution on developmental instability. Our analyses showed that concentration of heavy metals (Cd, Pb, Cu, Zn, Se) in leaves and roots of the seagrass were significantly higher in the polluted location than in relatively unpolluted locations. We found significant differences in TFA between different locations, showing that the method is sensitive enough to detect spatial differences even within a rather small water body, but these differences were not associated with a higher concentration of heavy metals, i.e. plants from the polluted location did not show higher TFA. Possibly, seagrass can store heavy metals in their tissues and protect their development from the toxic effect, or the effect of heavy metals in the natural environment is confounded by other environmental factors. At the same time, we found that plants from the polluted location had narrower leaves than in relatively unpolluted ones, which may be caused by heavy metals or associated factors.*

Keywords developmental instability, heavy metals, translational fluctuating asymmetry, *Zostera capricorni*

Introduction

Seagrass beds serve as nursery grounds for numerous ecologically and commercially important species of fish and invertebrates (den Hartog 1977; Kikuchi and Peres 1977; Gillanders 2006). Seagrass leaves and stems support numerous and abundant epiphytes, which are fed upon by small epifaunal organisms, such as amphipods and gastropods (Jernakoff and Nielsen 1998). Epifaunal organisms, in turn, provide food to the fishes foraging in the seagrass beds (Hemminga and Duarte 2000) and constitute an important link between primary producers, such as microalgae and detritus, and higher-level consumers (Kikuchi and Peres 1977; Edgar and Shaw 1995). Hence, seagrass meadows are

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essential in maintaining marine biodiversity (Duarte 2000; Hemminga and Duarte 2000). Moreover, the strap-like leaves of seagrass plants slow the overlying water and allow sediment suspended in the water to fall out into the seagrass meadow. The extensive rhizome systems of the seagrass then stabilize the underlying sediment (Bulthuis et al. 1984).

In Australia, there are about 30 different species of seagrass from 11 genera. *Zostera capricorni* Aschers is the most common species of Australian estuarine seagrass that is restricted to sheltered areas in estuaries and semi-enclosed embayments (Wood 1959; Kirkman 1997). The study was conducted at Lake Macquarie of New South Wales, one of the largest estuaries in Australia, which is predominantly covered by *Z. capricorni*. However, seagrass beds in Lake Macquarie have experienced a significant reduction due to coastal development (King and Hodgson 1986).

The lake extends approximately 22 km in a north-south direction. It has a maximum width of 10 km and a maximum depth of 11 m, with an average depth of 8 m (Peters et al. 1999a). The total waterway area of Lake Macquarie is 120 km^2 and the catchments are 700 km^2 (Manly Hydraulics Laboratory 2002). It is separated from the ocean by a narrow entrance channel and sand-bars, resulting in poor tidal mixing (Spencer 1959). Despite this poor tidal exchange, the lake has a marine character (with salinity usually in the range of 25–33 ppt) because of minimal freshwater dilution from the fluvial inputs. Winds produce larger water mixing within the lake than do tides (Roy and Crawford 1984).

Lake Macquarie is surrounded by residential areas and used for a wide range of industrial activities, including power generation, smelting and extractive industries (Lake Macquarie City Council 1995). Urban development is extensive on the northern parts of the Lake Macquarie resulting in increased urban run-off while the southern parts are less developed, although urbanization has increased in the last decades (Batley 1987). Industrial development in the north is also more extensive than in the south. In addition to the effects of storm water, three major point sources have the potential to impact water quality within the north part of Lake Macquarie. These include: (a) Stockton Borehole Colliery (1 km north of the lake), which discharges sediments; (b) Edgeworth Sewage Treatment Plant (3 km north), which discharges nutrients such as phosphorus and nitrogen; and (c) Pasminco Metals-Sulphide Smelter (1.5 km north east), which discharges heavy metals (Environmental Resource Management 2000).

The Pasminco Cockle Creek smelter was the major contributor of the heavy metals that contaminated Lake Macquarie from 1897 until its operation ceased on 12 September 2003. The smelter site was located at the northern end of Lake Macquarie (the area surrounding Cockle Creek and Cockle Bay). Although the smelter has now ceased operations, the accumulated contamination of the site and surrounding areas remains a serious issue. The sediment of Cockle Creek and Cockle Bay contained high concentrations of lead, zinc, copper and cadmium, which were one to two orders of magnitude greater than in sediments from other parts of Lake Macquarie (Spurway 1982; Batley 1991). Seagrasses grow in small patches around the foreshore. This might be related with the high concentrations of heavy metals that inhibited the growth of seagrass. Their actual role, however, was unclear (Environment Protection Authority 2001).

Heavy metals can be incorporated into seagrass leaves and vascular tissue from either water column or sediments. In locations where elevated concentration of metals was suspected, seagrass leaves also contained an elevated concentration of metals. The presence of heavy metals in both water and sediment has been demonstrated to inhibit the growth of seagrass (Ward 1989). Moreover, toxic concentrations of metals inhibited metabolic activity and interfered with vital biochemical pathways, such as photosynthesis (Ralph and Burchett 1998).

In field conditions, however, seagrasses seem relatively resistant to substances that can poison other forms of marine life. For example, turtle grass *Thalassia* maintained usual population density in the highly contaminated sediment receiving effluent from a desalination plant, while the nearby echinoids were killed. At the same time, the photosynthetic activity of the turtle grass was depressed by 50% during 24-hr exposure to a 12% effluent in the laboratory (McRoy and Helffrich 1980). Seagrasses *Z. mucronata*, *Posidonia australis* and *P. sinuosa* were found growing at their usual depths in sediments with extremely high concentrations of cadmium, lead, and zinc near a lead-zinc smelter at Port Pirie, South Australia. Their distribution seemed normal, although their leaves contained high concentrations of metals (Ward et al. 1984).

The effect of metals on seagrasses in the field seems to be very complex and could be modified by other environmental factors. Little is known about how seagrasses respond to contamination leading to a poor understanding of tolerance/resistance mechanism (Ralph et al. 2006). It is essential to understand whether metal can kill, permanently damage or merely cause stress to the seagrass.

Morphological traits of seagrass, in particular the shape of its leaves, have been investigated for use as indicators of environmental quality. It was found that narrower leaves were developed in more stressful conditions (McMillan 1978; McMillan and Phillips 1979; Phillips 1980). Another morphological characteristic that can be used for assessing stress is fluctuating asymmetry (FA). FA represents the random deviations from perfect symmetry and usually increases under stressful conditions (Tracy et al. 1995; Kozlov et al. 1996; Anne et al. 1998; Hosken et al. 2000; Lens et al. 2000; Lens et al. 2002; Mal et al. 2002; Tan-Kristanto et al. 2003). By its nature FA represents random component of phenotypic variance standing on equal footing with genotypic and environmental components (Lajus et al., 2003). Fluctuating asymmetry has been proposed as a tool for monitoring the quality of the environment and is being considered as a sensitive monitor of stress (Tracy et al. 1995; Anne et al. 1998; Leung et al. 2003). It has been claimed to be impacted at concentrations less than those required to impact life history features (Anne et al., 1998; Hoffmann and Woods 2003). Moreover, this technique has been recommended because it is biologically relevant, non-destructive, and time- and cost-effective (Tracy et al. 1995).

Using fluctuating asymmetry at present, however, is limited almost exclusively to structures that possess bilateral symmetry. At the same time, there are also other types of symmetry, such as translational symmetry, when a particular translation does not change the object. This type of symmetry was also proposed as potential measures of developmental instability (Graham et al. 1993). Although common in living organisms, the structures with non-bilateral symmetries are rarely used for analyzing symmetry (Freeman et al. 2003). The expansion of the developmental instability technique to structures possessing these types of symmetry would considerably increase the range of organisms in which it can be measured.

Fluctuating asymmetry measured on traits possessing translational symmetry is often called "translational asymmetry" (Alados et al. 2001; Sinclair and Hoffmann 2003; Tan-Kristanto et al. 2003; Alados et al. 2006). We suggest that term "translational fluctuating asymmetry" is more appropriate in this case because asymmetry of translationally symmetrical traits, like asymmetry of bilaterally symmetrical traits, can be both fluctuating (i.e. randomly deviating from perfect translational symmetry) and directional (i.e. have systematic deviations from perfect translational symmetry). Directional asymmetry is manifested, for instance, in the decrease of internode distances from the root to the top of the plant. Using the "translational asymmetry" together with the term "fluctuating asymmetry" unintentionally suggests the different nature of the phenomena, although indeed the same characteristic, namely developmental instability, is measured. The only difference is the type of symmetry from which the random deviations take place. The term "translational symmetry" is in the same manner incomplete as the term "bilateral symmetry," because both can be either fluctuating or directional. When authors use "fluctuating asymmetry," they by default mean that it is analyzed on bilaterally symmetrical traits. Analyses of fluctuating asymmetry on translationally symmetrical traits is considered a relatively new method, although such analyses were already performed a long time ago (Astauroff 1930), and it is important to point out that in this study we also deal with deviations from translational and not bilateral symmetry.

The longitudinal veins on the leaves of *Z. capricorni* are interconnected by the lateral veins at regular intervals (Kuo and den Hartog 2006). This character clearly shows translational symmetry. Thus, translational fluctuating asymmetry measurement in *Z. capricorni* is a measure of deviation from perfect translational symmetry, which is a translation along a certain straight line over some interval.

There were two main goals in this study: first, to develop an effective technique of measuring translational fluctuating asymmetry on seagrass, *Z. capricorni*; and second, to determine if there are differences in translational fluctuating asymmetry and leaf dimension characters between locations with different heavy metal loading.

Materials and Methods

Sampling Locations

Choice of sampling locations was based on the previous studies about heavy metal concentrations in the sediment of Lake Macquarie (Spurway 1982; Roy and Crawford 1984; Batley 1987; Peters et al. 1999b; Kirby et al. 2001; Roach 2005). We sampled in one polluted location (Cockle Bay), which had the highest concentration of heavy metals in the sediment, and six relatively clean locations (Fennel Bay, Killaben Bay, Wangi-Eraring Bay, Myuna Bay, Bonnels Bay, and Wyee Bay) (Figure 1). Depth ranged from 40 to 60 cm in all locations and types of sediment were sand and mud. In each location, we sampled three sites at distance 100–200 m. Within each site, 20 individual plants not closer than 3 m from each other were collected.

Measurement of Leaves

We selected two individual leaves with minimum epiphytic growth from each plant. The length of leaf was measured from the base to its tip. The width of leaf was measured from one side of the margin to the other side in its widest section. These measurements were taken using a mm-ruler. Then the leaves were cut up to about 11 cm from the leaf-sheath to the tip of the leaf and cleared of epiphytes by scraping the leaf surface. After that the leaves were scanned (using Epson Perfection 3170 Photo at resolution 1200 dpi). To account for measurement error, after taking the first image, each leaf was turned over and scanned again (Stige et al. in press).

The analysis of images (two from each leaf) was performed with "Image Tool" software (University of Texas Health Science Centre, San Antonio, Texas; freely available at ftp://maxrad6.uthscsa.edu). We placed 11 dots along the medial vein of the leaf in places

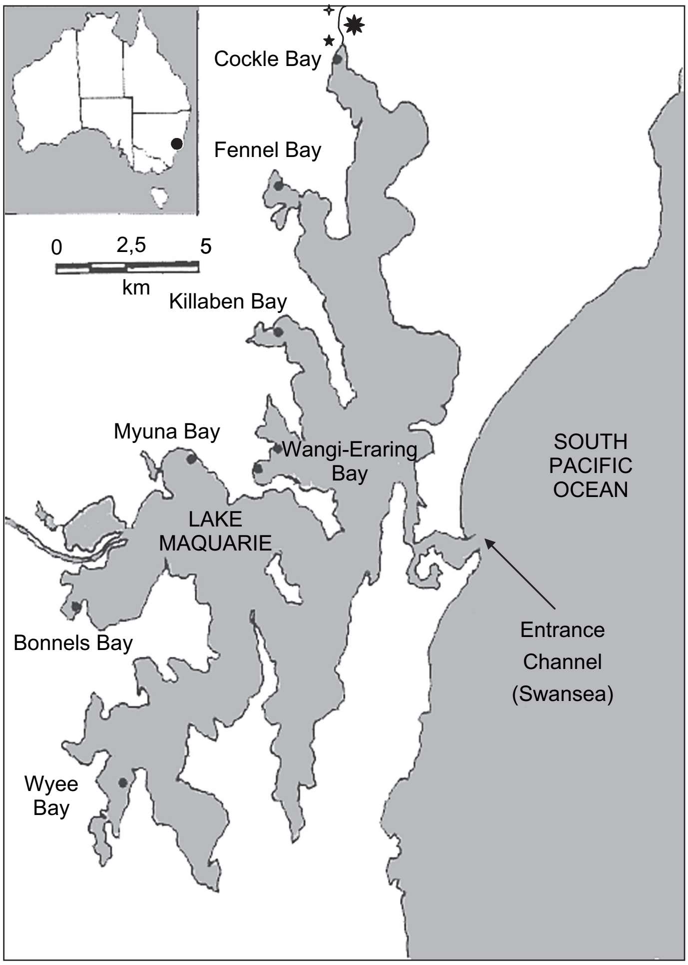

Figure 1. Sampling locations from north to south along the western part of Lake Macquarie, New South Wales, Australia. The point sources of heavy metal were indicated with symbols: + Colliery ★ Sewage treatment plant ✸ Lead-zinc smelter.

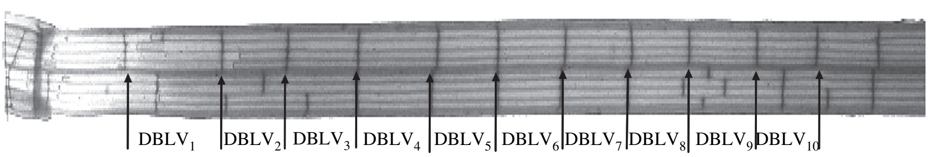

Figure 2. Translational fluctuating asymmetry (TFA) measurement of *Z. capricorni* leaf. Arrows indicate interception points between lateral veins of the upper half of the leaf and medial veins. Distances between these points are distances between lateral veins (DBLV). Ten distances were measured from each specimen.

of interception with lateral veins and obtained coordinates of these dots. Based on these coordinates, we calculated 10 distances between the lateral veins (DBLV) from each individual leaf (Figure 2).

Heavy Metal Analyses

Prior to the heavy metal analyses, seagrass tissue was washed with lake water to remove residual sediment and other debris, because previous studies showed that rinsing seagrasses with distilled water caused premature leaching of metals and other cations (Ledent et al. 1995). Epiphytes were removed by scraping them from the leaf surface. The seagrass tissue was blotted dry with paper towels and set out at room temperature until partially dry. Then, the seagrass tissue was oven dried at 60ºC for 48 h followed by separation into leaf blades and roots/rhizomes. The individual seagrass plants from each sampling site were combined and three sub-samples of about 0.1 g (dry wt) leaves or root/rhizomes material were placed into 50 ml beakers. All glassware was acid-washed in 5% nitric acid prior to the use for metal analyses. Ten milliliters of concentrated nitric acid was added to each beaker before it was placed on a hot plate at 85 – 95°C with a watch-glass on top of it. The samples were allowed to digest for several hours before 1 ml hydrogen peroxide was added step-wise, while the samples remained on the hot plate for several more hours. Upon completion of the digestion, the samples were allowed to cool, and then filtered into 50 ml volumetric flasks using Whatman no. 5 filter paper. The solution was made up to 50 ml using Milli-Q deionised water. Samples solutions were filtered through Sartorius 0.45 µm acetate membrane filters and stored in 10 ml plastic centrifuge tubes. The sample solutions were analyzed using a Thermo Electron High Resolution Inductively Coupled Mass Spectrometer (ICP-MS; Element 2) to determine metal concentrations.

Analytical quality control was performed with a reference material, estuarine sediment. As there is no certified reference material for seagrass, samples of Z. *capricorni* from Lake Macquarie that has been dried, mixed and ground to less than 0.8 µm was used. Spike recoveries of 0.1 ppm of solution (Cr, Cu, and As) were performed. Reference material and spike seagrass material were digested with each analytical batch to check recovery rates. In addition to check reference sediment material, the certified reference material PACS-2 (National Research Council Canada) was analyzed. The recovery rates obtained for sediment were: 60.75% for Cu, 84.83% for Cd, 75.46% for Pb, 130.43% for Se, and 84.73% for Zn, while the recovery rates for seagrass were 92.55 ± 6.77% for Cr, 82.26 ± 5.32% for Cu, and 108.11 ± 7.26% for As.

Statistical Analyses

Asymmetrical analysis of variance (ANOVA) was used for all variables as we wanted to compare between one location that was purportedly *impacted* and more than one control locations. This analysis was designed to deal with the environmental impact assessment when no data have been obtained before the purported impact and, thus, only *after* data are available, so called ACI (After-Control/Impact) (Underwood 1991; 1992; 1993; 1994). More specifically, in the absence of *before* data, it may be possible to detect consistent differences between one or more impact locations and several control or reference locations. Glasby (1997) provided a detailed description of how to deal with asymmetrical data and a discussion of the problems associated with detecting impacts when only *after* data are available.

The differences in concentrations of heavy metals (Cd, Pb, Cu, Zn, Se) between polluted and control locations were identified with the asymmetrical ANOVA: Location (random, with one polluted and six controls) and Site (three sampling sites, nested in Location), with three replicate measurements at each site. The main indication of impact would be a significant difference between Cockle Bay and control locations (CB *vs* Control in the analysis table).

Leaf-length, leaf-width, and ratio between leaf-width and leaf-length were calculated and also compared between polluted and control locations with an asymmetrical analysis of variance with factors: Location (random, with one polluted and six controls) and Site (three sampling sites, nested in Location), with 20 replicates at each site. The similar experimental design was used for average DBLV with 40 replicate measurements at each site.

Translational fluctuating asymmetry (TFA) data were analyzed using three-factor asymmetrical analysis of variance (ANOVA): Location (random, one polluted and six controls), Site (three sampling sites, nested in Location), and Leaf (two leaves), with 20 replicate measurements per combination of factors. Moreover, TFA data were correlated with metals concentrations in both leaves and roots/rhizomes of seagrass using Spearman's correlation analysis.

The initial and repeat measurements of subset data (20 plants per location) were analyzed to determine whether the variation in non-directional asymmetry (i.e. TFA) was significantly larger than the measurement error. This was done through a two-way ANOVA with DBLV and individual plant as random factors, which also test for directional asymmetry (DA) (Palmer 1994).

As fluctuating asymmetry is deviation from perfect symmetry (in this case translational symmetry), we first needed to perform a standardization to adjust our data to perfect translational symmetry. This standardization was needed because there was a variation in DBLV for each leaf. To standardize, we first divided each individual DBLV to average DBLV for each leaf to adjust changes from first to last DBLVs, and second, each DBLV was divided by an average length of respective DBLV for each site to account for systematic variation in DBLV within a leaf.

Results

Heavy Metals in Seagrass Tissues

Concentration of heavy metals in the roots/rhizomes of seagrass were highly correlated with the concentrations in its leaves with exception for Cd (r ranged from 0.84 to 0.99, $p < 0.01$ for other metals), while correlation coefficient for Cd was 0.15 ($p > 0.05$). Roots in most cases contained higher concentrations of metals than leaves with the most pronounced difference observed for lead (Tables 1 and 2). Spatial variation in heavy metal concentrations was observed both within and between locations, with significantly higher concentrations in samples from Cockle Bay (polluted location) compared to controls (Tables 1 and 2). At the same time, there were notable differences between different sites within Cockle Bay. Patterns of differences varied from metal to metal, but in all metals at least one site within the Cockle Bay exhibited a considerably higher concentration of metal than in all other locations (Table 2).

Leaf Dimension Characters

The size of *Zostera* leaves in term of their length and width varied significantly among locations ($F_{6,14}$, $p < 0.01$), but the variation in these variables was more pronounced among the control locations and among the site within control locations. Overall, *Zostera* leaves in Cockle Bay were longer than in control locations though the differences were not significant. The width of the leaves varied among locations and no further differences between polluted and control locations were found (Figures 3a and 3b). Variation in leaf dimensions was not correlated with depth.

Table 1
Heavy metal concentration (µg g^{-1} dry wt) in seagrass *Z. capricorni* from Lake Macquarie, New South Wales, Australia

Location	Section	Cd	Pb	Cu	Zn	Se
Cockle Bay	Root	20.2 ± 5.4	211.7 ± 44.5	84.1 ± 33.2	592.4 ± 138.9	10.6 ± 7.2
	Leaf	6.1 ± 2.0	148.4 ± 30.6	52.1 ± 15.9	396.5 ± 90.6	7.3 ± 5.5
Fennel Bay	Root	3.1 ± 0.9	26.5 ± 2.0	17.4 ± 2.1	116.2 ± 14.1	0.1 ± 0.04
	Leaf	2.1 ± 0.5	20.2 ± 2.7	13.8 ± 1.3	170.9 ± 13.2	0.1 ± 0.04
Killaben Bay	Root	5.6 ± 0.5	42.6 ± 18.9	19.7 ± 1.7	152.8 ± 11.7	2.0 ± 0.7
	Leaf	4.2 ± 0.4	6.2 ± 0.4	13.5 ± 1.5	134.4 ± 19.2	0.6 ± 0.1
Wangi-Eraring Bay	Root	3.3 ± 0.6	4.1 ± 0.4	15.3 ± 2.2	65.7 ± 15.1	0.2 ± 0.05
	Leaf	4.4 ± 0.4	3.4 ± 0.2	15.0 ± 1.6	115.4 ± 25.1	0.04 ± 0.02
Myuna Bay	Root	3.0 ± 0.5	4.1 ± 0.5	22.3 ± 2.4	63.9 ± 4.6	0.9 ± 0.06
	Leaf	6.6 ± 1.1	4.1 ± 0.6	18.6 ± 2.6	119.6 ± 20.1	1.2 ± 0.2
Bonnels Bay	Root	4.2 ± 0.5	7.4 ± 0.7	17.2 ± 1.7	104.4 ± 10.3	1.05 ± 0.2
	Leaf	4.8 ± 0.3	7.3 ± 1.1	17.4 ± 0.6	154.8 ± 18.1	0.8 ± 0.05
Wyee Bay	Root	5.8 ± 0.7	5.9 ± 0.8	20.9 ± 2.7	65.2 ± 6.4	1.3 ± 0.1
	Leaf	8.4 ± 1.1	5.5 ± 0.8	19.7 ± 1.8	128.2 ± 6.6	1.3 ± 0.1

Table 2
Summary of asymmetrical analysis of variance (ANOVA) results comparing heavy metals concentrations in *Z. capricorni* between polluted (CB) and control locations

		Cadmium (Cd)		Copper (Cu)		Lead (Pb)		Zinc (Zn)		Selenium (Se)	
Source of variation	DF	MS	F	MS	F	MS	F	MS	F	MS	F
Section	1	47.40	0.91 *ns*	1420.29	3.07 *ns*	7387.65	11.36**	2256.36	0.10 *ns*	14.48	2.49 *ns*
Locations	6	220.08	4.52**	6633.61	1.99 *ns*	74532.28	6.92**	378299.98	4.54**	175.55	1.02 *ns*
CB *vs* Control	1	1124.27	28.65**	39329.25	416.26***	438703.81	258.37***	2210129.82	185.20***	1025.15	181.97***
Control	5	39.24	2.80 *ns*	94.48	0.87 *ns*	1697.97	5.25**	11934.02	4.48*	5.63	5.20**
Sites (Locations)	14	48.69	2.99***	3325.85	5.84***	10774.25	11.75***	83349.79	10.39***	172.51	4.19***
Sites (CB)	2	256.66	18.30***	22632.43	209.40***	73478.07	227.06***	567461.58	212.97***	1201.09	1109.49***
Sites (Control)	12	14.02	6.08***	108.08	5.33***	323.61	1.25 *ns*	2664.49	1.56 *ns*	1.08	3.34 *ns*
Section × Locations	6	157.91	3.02*	583.24	1.26 ns	2793.19	4.30*	39940.88	1.69 *ns*	6.97	1.20 *ns*
Section × Sites (Locations)	14	52.24	3.21***	462.99	0.81 ns	650.10	0.71 *ns*	23630.06	2.94**	5.81	0.14 *ns*
Residuals	84	16.28		569.43		917.27		8024.28		41.13	
Residuals (CB)	12										
Residuals (Control)	72										
Total	125										

ns – not significant; * - significant at $p < 0.05$; ** - significant at $p < 0.01$; and *** - significant at $p < 0.001$.

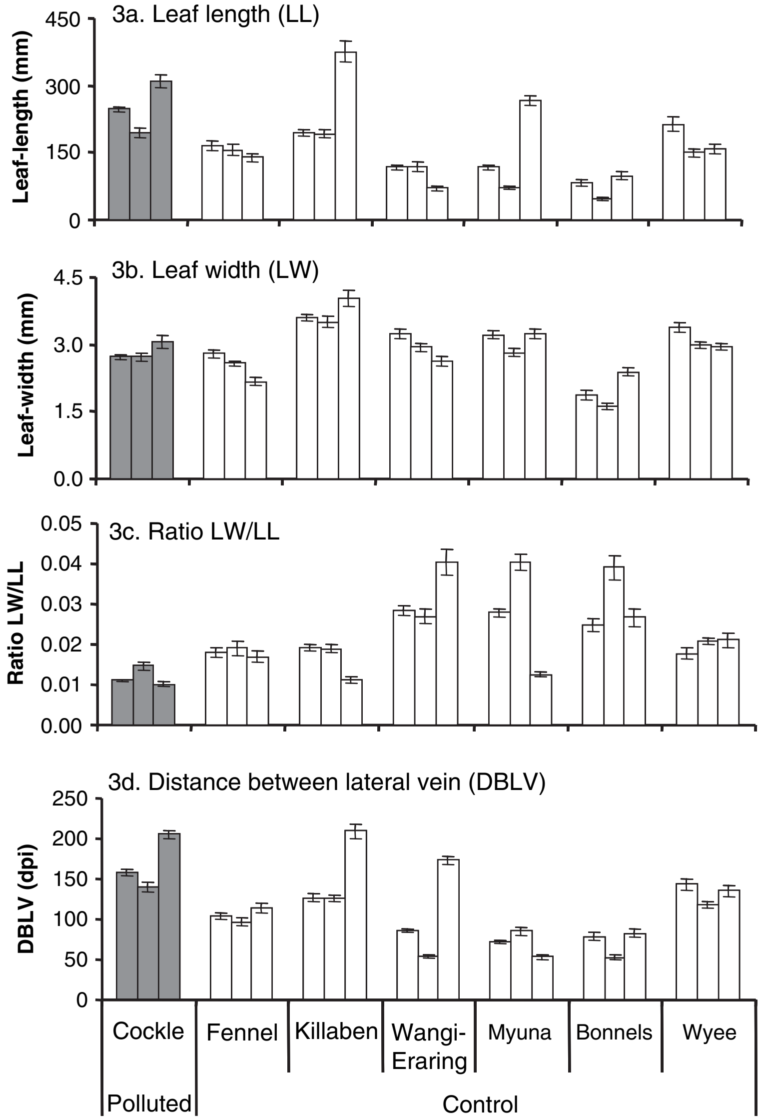

Figure 3. Leaf-length (3a), leaf-width (3b), ratio leaf-width and leaf-length (3c), and distance between lateral vein (DBLV; 3d) of *Z. capricorni* in comparison between polluted and control locations.

Ratio of leaf-width and leaf-length of *Zostera* leaves were significantly smaller in Cockle Bay (the polluted location) than in control locations (Table 3, Figure 3c). There was more variation in the dimension of the leaves among the sites within control locations than among the sites within the polluted location.

Patterns of Variation in Distances between Lateral Veins DBLV and Translational Fluctuating Asymmetry (TFA)

Distances between lateral veins (DBLV) decreased from the base to the top of the leaf. The pattern of decrease was most pronounced from the first to the fifth DBLV (Figure 4).

Table 3
Summary of asymmetrical analysis of variance (ANOVA) results comparing leaf dimension of *Z. capricorni* between polluted (CB) and control locations

Source of variation	DF	MS	F
Locations	6	7.15	4.14*
CB *vs* Control	1	20.16	7.52*
Control	5	4.55	2.39 *ns*
Sites (Locations)	14	1.73	22.80***
Sites (CB)	2	0.68	0.36 *ns*
Sites (Control)	12	1.90	23.61***
Residuals	399	0.08	
Residual (CB)	57	0.05	
Residual (Control)	342	0.08	
Total	419		

ns – not significant; * - significant at $p < 0.05$; *** - significant at $p < 0.001$.

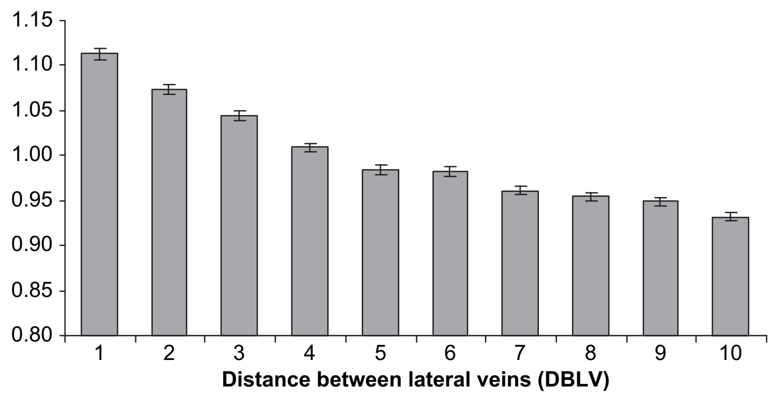

Figure 4. Pattern of variation in distance between lateral vein (DBLV) of *Z. capricorni* leaves. Change axes designation: X-axis – ordinary number of DBLV (starting from the root) and Y-axis – length of respective DBLV.

On average, each next DBLV was about 2,2 % smaller than the previous one. The average DBLV was significantly bigger in Cockle Bay compared to control locations ($F_{1,17} = 5.27$, $p < 0.05$; see also Figure 3d). There was a significant positive correlation between the length of the leaf and the size of DBLV (Spearman's correlation, $p < 0.0001$), i.e. differences in length of leaves were explained by differences in size of DBLV, rather than by their number.

Standardized DBLV (see Material and Methods section) were checked for directional asymmetry, i.e. to check if the standardization was correct, using two-way ANOVA. The test revealed that factor *DBLV* was not significant ($F_{9,1251} = 0.95$, $p > 0.05$), indicating that there was no directional asymmetry (DA). The non-significance of factor *Individual*

Table 4
Summary of asymmetrical analysis of variance (ANOVA) results comparing translational fluctuating asymmetry (TFA) of *Z. capricorni* leaves between polluted (CB) and control locations

Source of variation	DF	MS	F
Location	6	0.61	6.43**
CB *vs* Control	1	0.42	0.65 *ns*
Control	5	0.65	6.27**
Site (Location)	14	0.10	0.82 *ns*
Site (CB)	2	0.04	0.43 *ns*
Site (Control)	12	0.10	0.91 *ns*
Leaves	1	0.00	0.00 *ns*
Location × Leaves	6	0.21	0.98 *ns*
Leaves × Site (Location)	14	0.21	1.82*
Residuals	798	0.12	
Residuals (CB)	114	0.13	
Residuals (Control)	684	0.11	
Total	839		

ns – not significant; * - significant at $p < 0.05$; ** - significant at $p < 0.01$.

($F_{139,1251} = 0.01$, $p > 0.05$) means that the variation of DBLV was not different among individual plants within the site. Deviation from perfect translational symmetry (i.e. translational fluctuating asymmetry) was shown by a significant interaction of factors *DBLV x Individual* ($F_{1251,1400} = 2.50$, $p < 0.0001$). The significant deviation from the perfect translational symmetry ($p < 0.0001$) in this case was larger than measurement error. Repeated measures of translational fluctuating asymmetry were positively correlated ($r = 0.987$, $p < 0.01$) indicating that measurement error was negligible.

There was no significant differences in TFA between first and second leaves, and among sites within a location, but differences between locations were significant ($F_{6,14} = 6.43$, $p < 0.01$). TFA significantly varied among the control locations with the highest values in Killaben Bay and the lowest in samples from Fennels Bay. There was no significant increase in TFA of *Zostera* leaves from the polluted location (Table 4, Figure 5). There was also no correlation found between metals concentrations in seagrass tissues and the TFA (Spearman's correlation, $p > 0.05$).

Discussion

Concentrations of heavy metals in seagrass, *Z. capricorni* were significantly higher in Cockle Bay (the polluted location) compared to control locations, reflecting the corresponding concentrations of heavy metals in the sediments of Lake Macquarie recorded in the previous studies (Spurway 1982; Roy and Crawford 1984; Batley 1987; Peters et al. 1999b; Kirby et al. 2001; Roach 2005). This result is in line with other studies of the relationships between metal levels in the environment and in seagrasses (Brix et al. 1983; Ward 1987; Ward 1989; Malea et al. 1994; Sanchiz et al. 2000; Campanella et al. 2001; Filho et al. 2004). Therefore, given a simple correlation between metal levels in their

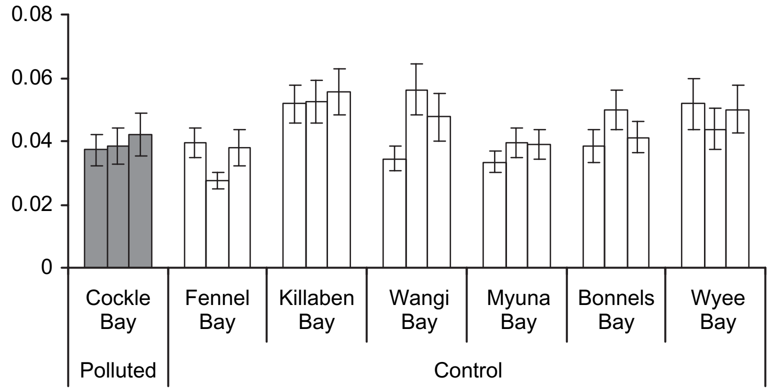

Figure 5. Translational fluctuating asymmetry of *Z. capricorni* leaves in comparison between polluted and control locations.

tissue and average metal levels in its environment, seagrasses can be used as an indicator for heavy metal pollution (Phillips 1977).

Experimental exposure of seagrass to concentrations of heavy metals in water from 0.5 to 5 ppm for Pb, Cu, Cd and Zn had an effect on the photosynthetic activity, structural characteristics of leaves and shoots, and on seagrass growth (Conroy et al. 1991). However, seagrass exposed to higher concentrations of heavy metals (18.4 ppm of Cd, 406 ppm of Pb and 1270 ppm of Zn) in the field did not show evidence of growth inhibition (Ward et al. 1984; Ward 1987).

In this study, however, seagrass leaves from the more polluted location were longer and had smaller width/length ratio than the leaves from control locations. Phillips (1980) suggested that leaf dimension characters are plastic and vary according to the environment where the plants live. For example, *Thalassia* populations from shallow and turbid bays had narrow leaves and those from clear water have wider leaves; *Halodule* populations with frequent exposure to air at low tides tended to produce narrow leaves and those that were not exposed at low tides tended to produce wider leaves; *Zostera* populations from intertidal areas produced narrow leaves and those in subtidal habitats produced broader leaves (McMillan and Phillips 1979; Phillips 1980). Moreover, turbid water and variable salinity and temperature of the shallow bays correlated with the presence of narrow-leaved plants, and the clear water and relatively constant salinity and temperature of the bays correlated with the broader-leaved populations (McMillan 1978).

Interestingly, the difference in DBLV showed even more pronounced differences between polluted and control locations. DBLV was clearly correlated with length of leaf and therefore increase in leaf length is caused by increase of DBLV rather than the number of DBLV. Possibly, the increase of DBLV reflects a response of *Zostera* to heavy metals on a tissue level; however, other studies are needed to investigate the utility of this morphological response as a marker for the effect of heavy metals on seagrass.

The difference in the leaf dimension of seagrass between polluted and unpolluted locations in this study might be related to the high concentration of heavy metals in the sediment or might be a response to the high turbidity and high nutrient content in the polluted site. The high levels of suspended solids, nitrates and orthophosphates in the water at the northern side of Lake Macquarie have been observed previously (Conroy et al. 1991). This suggests that heavy metals may have an effect on seagrass morphology, although the effect should be demonstrated by manipulative studies (i.e. transplantation experiments).

Traditionally, fluctuating asymmetry is most frequently studied on bilaterally symmetrical traits, although translational symmetry is known to be suitable for such analyses (Graham et al. 1993). Recently, several works analyzing translational fluctuating asymmetry and showing that it can be used as a good indicator of environmental stress on plants, were published (Alados et al. 2001; Sinclair and Hoffmann 2003; Tan-Kristanto et al., 2003; Alados et al. 2006). *Zostera* with their wide distribution range, important role in coastal ecosystems and vulnerability to human-induced changes, represents one of such objects.

The method developed in this study showed high resolution in terms of discriminating geographically distinct samples. Samples situated at distances of 3–5 km showed significant differences in fluctuating asymmetry, although the analyses showed homogeneity of the samples from distances of hundred meters. Most likely, variation in environmental factors causes variation in fluctuating asymmetry, the but effect of genotypic factors, namely adaptation to local environmental conditions, may also be important.

None of the factors that we controlled in this study, such as heavy metal concentration, depth, and type of sediments, showed a significant effect on fluctuating asymmetry. Thus some uncontrolled factors probably cause the observed heterogeneity. Among them can be nutrient availability, light intensity, and turbidity of water. The polluted location chosen for this study was heavily polluted both by heavy metals and urban run-off. Therefore, the effect of heavy metals on seagrass might have been confounded by other uncontrolled factors (in this case, nutrient concentrations and level of water turbidity). The interaction of heavy metals and other stress factors (e.g. high nutrient level and turbidity) on seagrasses have not been studied so far (Ralph et al. 2006). According to the suggestion of Conroy et al (1991), heavy metals and nutrients may affect seagrass growth in opposite directions. The lack of differences in developmental instability in our study might be due to the fact that the negative effect of heavy metals and the positive effect of nutrients compensated each other.

This is supported by the results of our study on other seagrass species, *Halophila ovalis*, from the same locations in Lake Macquarie. Fluctuating asymmetry of *H. ovalis* from the most polluted location of the lake was not higher than in relatively unpolluted locations (Ambo-Rappe et al. 2007), but in laboratory experiments, fluctuating asymmetry of the seagrass increased under concentrations of Cu and Pb similar to those found in the lake water (Ambo-Rappe et al. unpublished data).

Tracy et al. (1995) also found no clear link between fluctuating asymmetry and environmental stress in an aquatic plant, *Ceratophyllum demersum*. The authors suggested that the absence of roots in this plant make it less exposed to the principal location of pollutants (in the sediments). Tan-Kristanto et al. (2003) found significantly lower trichome asymmetry of *Arabidopsis thaliana* when exposed to cadmium. They suggested that there is a possibility that heavy metal exposure induced or activated enzymes, such as phytochelatin synthase, in the plant that protects plant development from further damage, leading to a decrease in asymmetry rather than the expected increase. Fluctuating asymmetry is, therefore, not simply a function of pollution but mediated by other factors.

It also should be taken into account that seagrasses seem to be able to accumulate high concentrations of metals, store them in a special compartment where they have little metabolic effect (Ward 1989). Mechanisms of this phenomena in algae, which can be similar to those on seagrass are discussed by Pinto et al (2003).

Moreover, seagrass in Lake Macquarie, especially at the northern side, might have developed genetic tolerance to metals due to a long-term (more than 100 years) exposure from the smelter. Plants can increase a tolerance to metal exposure as a result of natural selection, where selection pressure favors the tolerant genotype. The tolerant seagrass populations from heavily developed estuaries were less sensitive to the metal contamination compared to seagrasses from a less developed estuary (Macinnis-Ng and Ralph 2004).

Therefore, observed differences in morphological characteristics of seagrass, *Z. capricorni*, from different locations in Lake Macquarie may be explained by combined effects of different environmental factors. In Cockle Bay, the high nutrient content might have promoted seagrass growth (including the increase in width and length of leaves, and increase of DBLV on tissue level), but high water turbidity and also possible high heavy metal content might have retarded the growth resulting in narrow leaves. Thus, there is a possibility that leaf dimension and tissue structure of seagrass, *Z. capricorni*, could be used as an indicator of environmental stress in estuarine system. However, the opposite effect of different environmental forces can also result in a complicated pattern of translational fluctuating asymmetry. The method used in our study is sensitive enough to detect spatial differences between populations, but to interpret these differences and to estimate utility of translational fluctuating asymmetry as a marker of environmental stress caused by heavy metals on *Zostera*, additional experiments are necessary.

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