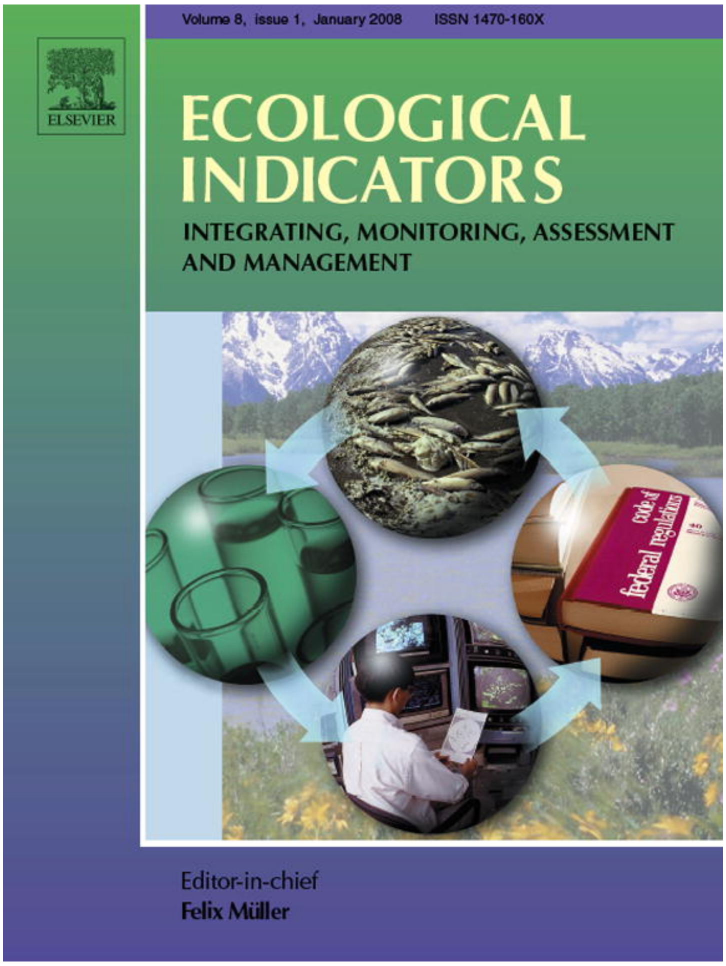
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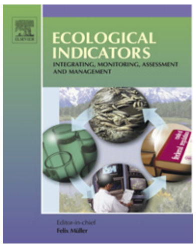

Notes

Increased heavy metal and nutrient contamination does not increase fluctuating asymmetry in the seagrass *Halophila ovalis*

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ABSTRACT

Fluctuating asymmetry (random differences between symmetric structures, FA) is one of the stress indices used recently to assess a subtle effect of environmental degradation on organisms and is expected to increase under stress conditions. In this study, we developed an original technique of measuring FA in seagrass, *Halophila ovalis*. We analysed five metric and meristic characters on leaves of the seagrass from a polluted and several control locations in a lagoon in Eastern Australia. The seagrass was sampled from three sites at each location. The analyses revealed significant spatial heterogeneity of samples in fluctuating asymmetry with the highest variability was observed among sites. There was no increase in FA of *H. ovalis* from polluted location. Possible explanations suggest that whether existing concentrations of heavy metals do not cause developmental stress in seagrass or their effect is compensated or even surpassed by effect of uncontrolled factors.

1. Introduction

The sediments of Lake Macquarie, an estuarine lake in New South Wales, Australia are contaminated with trace metals such as cadmium, lead and zinc (Roy and Crawford, 1984). The contamination is higher in the northern side of the lake due to more extensive industrial development (Batley, 1987; Environmental Resource Management, 2000). Toxic heavy metals have been accumulated in Lake Macquarie since 1897–2003 due to the operation of the lead–zinc smelter, resulting in the contamination of the northern reaches (Cockle Creek and Cockle Bay) of Lake Macquarie with copper, lead, zinc and cadmium. Batley (1987) reported elevated concentrations of selenium in surface sediments in Cockle Bay and in the roots and rhizomes of seagrasses. Our analyses carried out in 2004 also revealed higher concentration of cadmium, lead, copper, zinc and selenium in seagrass species, *Zostera capricorni*, from Cockle Bay compared to other locations (Ambo-Rappe et al., unpublished data). The heavy metal concentrations reduce southward through the lake indicating that the lead–zinc smelter was a major source of contaminants (Roy and Crawford, 1984).

Z. capricorni and *H. ovalis* are common seagrass species in Lake Macquarie. They notably declined for 700 ha over the past 20–25 years period (King and Hodgson, 1986), however, a role of heavy metals in this decline is unclear.

In the present study, we have analysed fluctuating asymmetry (FA) on *H. ovalis* to test whether given pollution

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levels cause increase of fluctuating asymmetry. FA is a measure of developmental stability and is frequently used now to assess environmental stresses (Hoffmann and Woods, 2003; Leung et al., 2003). This is the first study of FA on *H. ovalis* thus we paid much attention in developing a technique of measuring FA on this species.

2. Methods

H. ovalis were sampled during November 2003–January 2004 in seven localities in Lake Macquarie, New South Wales, Australia (151°66′E, 33°08′S). The most northern locality (Cockle Bay) was the most polluted, other localities (Fennel Bay, Killaben Bay, Wangi-Eraring Bay, Myuna Bay, Bonnels Bay and Wyee Bay; southward of Cockle Bay) were relatively unpolluted. Distances between localities were 2–4 km. Within each locality we sampled three sites at a distance of 100–200 m from each other. From each site, we collected 20 individual plants separated by no less than 3 m from each other to avoid repeated sampling of the same individual. Two leaves were chosen from each plant based on the clearness of the vein. Then, the leaves were arranged in a standard manner (taking into account upper and lower surface of the leaf) and scanned using scanner (Epson Perfection 3170 Photo, 1200 dpi). To estimate measurement error, after first scan, each leaf was turned out and scanned the second time (following Stige et al., in press).

The measurements on the images were taken using 'Image Tool' software (freely available at ftp://maxrad6.uthscsa.edu). There were five traits measured for leaf asymmetry of *H. ovalis*: number of intersections of lateral veins with the peripheral vein (Trait 1), number of intersections of lateral veins with the central vein (Trait 2), length of peripheral vein (Trait 3), length of central vein (Trait 4), length of first lateral vein (Trait 5). Length of veins was measured as sum of distances between intersections of lateral veins with peripheral or central veins. Additional measurements were also done for leaf length, which is a measurement of the length of the midrib; leaf width, which is the distance from the left to the right margin of the leaf's widest point.

Before comparing samples by FA, we performed tests for directional asymmetry (DA) and antisymmetry that might occur together with FA and measurement error (ME) (Palmer, 1994). ME was evaluated for each trait by conducting two-way ANOVA with side and individual as random factors. The ANOVA also tests the presence of DA, the significance of asymmetry due to the size/shape among individuals, the significance of non-directional asymmetry, which refers to FA or antisymmetry (Palmer and Strobeck, 1986; Palmer, 1994). To test for antisymmetry, we checked departure from normality of the distribution of "left minus right" within each trait using Kolmogorov–Smirnov test (Sokal and Rohlf, 1995). We also checked the data for statistical outlier that is a common source of skew or leptokurtosis in studies of FA (Palmer, 1994).

For all traits, we calculated FA index based on unsigned left minus right characters. Asymmetrical ANOVA was undertaken to compare the FA between polluted and relatively unpolluted locations as we had only one polluted location with six controls (Underwood, 1994).

3. Results and discussion

The two-way ANOVA revealed no significant difference between sides in Traits 1–4 ($F_{1,839}$; $p > 0.05$), indicating a lack of DA. Trait 5 showed DA ($F_{1,839}$; $p < 0.001$). In 785 out of 1680 comparisons, lateral vein on the left side was longer than on the right side. At the same time, in average, length of right veins exceeded length of left veins on about 0.5%. Given such complicated patterns of directional symmetry, Trait 5 was excluded from further analyses.

From the two-way ANOVA test, we also found that the significance of asymmetry is due to the size/shape among individuals ($F_{839,839}$; $p < 0.001$). Moreover, we found positive correlation between leaf asymmetries and leaf length which indicated an increase in leaf asymmetry with increasing size (Spearman's correlation, $p < 0.001$). A dependence of asymmetry on individual size can influence inferences made in studies of developmental stability (Palmer, 1994), therefore, we made a size-correction by standardising the asymmetries data with leaf length. After applying the correction, Spearman's correlation was performed again and no correlation was found.

There was a significant interaction of factors "individual × side" indicating the presence of non-directional asymmetry ($F_{839,1680}$; $p < 0.0001$). The measurement error explained only 7.1%, 7.3% and 7.4% of non-directional variation in Traits 1–3, respectively. Trait 4 was found to have very large measurement error (118%) and was excluded from further analyses.

Since non-directional asymmetry may relate to FA or antisymmetry or both, it is instructive to know if the antisymmetry takes a part on the non-directional asymmetry observed in this data. Kolmogorov–Smirnov test revealed that (L–R) distributions of Trait 3 did not deviate from normal distribution, but significant deviations were found for Traits 1 and 2. Significant positive kurtosis was detected for (L–R) distribution of these traits ($p < 0.001$) indicating leptokurtic distribution (Sokal and Rohlf, 1995). Therefore, care should be taken to show that inferred differences in developmental stability among samples are not confounded by this factor (Palmer, 1994).

FA of the metric trait (Trait 3) significantly differed across locations and sites, but the variations were more pronounced among control locations and sites within control locations than between control and polluted locations and sites. No heterogeneity with respect to leaf for this trait has been found. Countable or meristic traits (Traits 1 and 2) did not show heterogeneity with respect to location, but varied notably among sites (significant for number of interception with peripheral vein). The variations with respect to leaf were found among locations for Trait 2 and among sites within locations for Trait 3. Moreover, there were no significant differences in fluctuating asymmetry of *H. ovalis* leaf between polluted and control locations (Table 1).

The results of the FA analyses in this study do not support an expectation of positive correlation between heavy metal contamination and developmental stress of seagrass in this lake. At the same time, we found significant heterogeneity of FA among locations and sites. Therefore, we have to postulate influence of factors other than heavy metal pollution on

Table 1 – Summary of asymmetrical ANOVA results comparing FA of *H. ovalis* between polluted (CB) and control locations

Source of variation	DF	Trait 1		Trait 2		Trait 3	
		MS	F	MS	F	MS	F
Location	6	0.02	1.01 ns	0.01	0.85 ns	0.01	3.61*
CB vs. control	1	0.00	0.01 ns	0.02	2.51 ns	0.01	1.24 ns
Control	5	0.02	1.05 ns	0.01	0.60 ns	0.01	3.25*
Site (location)	14	0.02	2.03*	0.01	1.56 ns	0.00	3.04***
Site (CB)	2	0.00	0.08 ns	0.00	0.26 ns	0.00	0.55 ns
Site (control)	12	0.02	2.33**	0.01	1.75 ns	0.00	3.25***
Leaf	1	0.04	4.44 ns	0.01	1.11 ns	0.00	0.14 ns
Location × leaf	6	0.01	0.60 ns	0.02	3.11*	0.00	1.15 ns
Leaf × site (location)	14	0.01	1.17 ns	0.01	0.96 ns	0.00	2.69***
Residual	1638	0.01		0.01		0.00	
Residual (CB)	234	0.01		0.01		0.00	
Residual (control)	1404	0.01		0.01		0.00	
Total	1679						

ns: not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

developmental instability. Similarly, Tracy et al. (1995) found no clear link between FA and environmental stress in *Ceratophyllum demersum* and explained this by absence of roots in this plant, which makes it less exposed to the principal location of pollutants (in the sediments). Other authors (Tan-Kristanto et al., 2003) also 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. FA is, therefore, not simply a function of pollution but also interacts with other environmental factors. In our case, while analysing differences in environmental conditions in Cockle Bay, one can see that higher concentration of heavy metals are accompanied by higher concentrations of nutrients (Manly Hydraulics Laboratory, 2002) as both resulted from anthropogenic activities in this area. They may have an opposite effect on development. While heavy metals cause stress, nutrients may facilitate metabolism and therefore compensate effect of heavy metals on the response of a general indicator of stress such as fluctuating asymmetry. Observations of growth of the plants in polluted versus unpolluted showed a similar or even a bit better individual growth rate of *Halophila* leaves in polluted compared to control locations (Fig. 1). Possibly, detrimental effect of heavy metals on growth is compensated by favourable effect of elevated nutrients. Thus, it is not surprising to find no significant increase of FA on this plant due to the metal pollution as FA is a predictor of fitness changes as well (Clarke, 1995).

Another possible reason for lack of increase in FA in polluted location in this study might an effect of natural selection leading to adaptation to live in polluted location. Seagrasses in Lake Macquarie, especially at the northern side, might have developed tolerance to metals due to a long time exposure from the smelter (more than 100 years). For example, the tolerant seagrasses populations from heavily developed estuaries were less sensitive to the metal contamination compared to seagrasses from least developed estuary (more pristine condition) (Macinnis-Ng and Ralph, 2004).

Different organs of plants exhibit multiple forms of symmetry. Consequently, many different estimates of FA can be made (Freeman et al., 2003). Moreover, different estimates of FA can display different sensitivities (Freeman

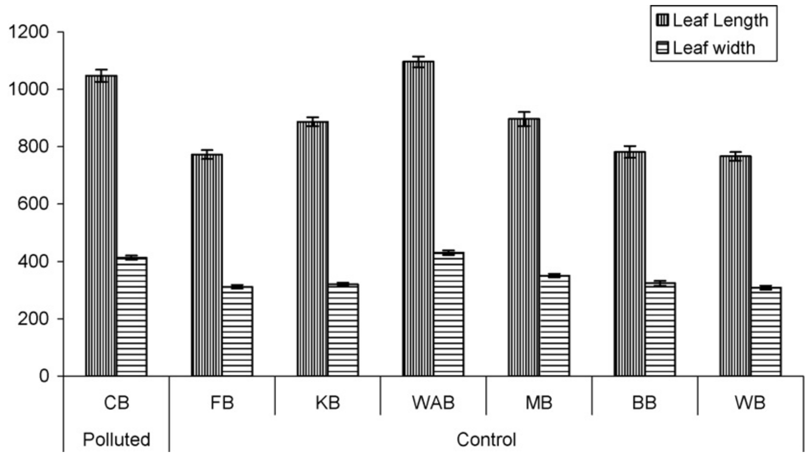

Fig. 1 – Leaf length and width of *H. ovalis* between polluted and control locations.

et al., 2005). Different characters may also exhibit different patterns of fluctuating asymmetry depending on their size (Lajus, 2001). Selection of certain traits to be used as estimates of asymmetry clearly depends on the knowledge of the biology of the species being studied (Freeman et al., 2005). This is unlikely, however, because measures of FA have been sensitive enough to detect variability among locations and sites.

Therefore, the following hypotheses can be suggested to explain why there is no positive correlation between heavy metal pollution and fluctuating asymmetry of *H. ovalis* in Lake Macquarie: (i) plants developed genetic resistance to heavy metal pollution through selection during more than a century of the smelter operation and (ii) effect of heavy metal pollution is rather weak and overlapped or compensated by uncontrolled factors such as nutrients, which notably show that not only FA, but also growth does not change in polluted location. An experimental study involving growing *H. ovalis* at different metal concentrations in the laboratory is necessary for determining whether heavy metal pollution itself could lead to increased FA in this plant.

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