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Full Length Research Paper

Heavy metal impact on growth and leaf asymmetry of seagrass, *Halophila ovalis*

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A major threat to the seagrass ecosystem worldwide, due to the growth of human population along the coastal environment, is pollution or contamination resulting from industrial and urban development. Although seagrass appears to be rather resistant to heavy metal contaminants, these substances may possibly harm some components of the seagrass and such responses have not been examined to a significant extent. Lead (Pb) and copper (Cu) was tested on seagrass, *Halophila ovalis*, to see whether the metals are environmental stressor on the seagrass. Reduced growth rate of the seagrass was observed both in Pb and Cu treatments. Leaf size of the plant also reduced as the metal concentrations increased and when the plants were exposed to the heavy metal for longer duration. An increased leaf asymmetry was more apparent at the 2 mg/L Cu treatment and no significant increases in fluctuating asymmetry were found in Pb treatment or in low levels of Cu treatment. Further discussion were made in view of selecting non-costly bioindicators of heavy metal contamination.

Key words: Bioindicators, fluctuating asymmetry, *Halophila ovalis*, heavy metals, seagrass.

INTRODUCTION

Seagrass habitats are subjected to stronger anthropogenic pressure than many other marine communities. This is particularly related to the close proximity of this habitat to human activities (Walker et al., 2001; Duarte, 2002). Heavy metals are significant environmental contaminants of seagrass systems (Pergent-Martini and Pergent, 2000). 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 (Ambo Rappe et al., 2007).

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). Therefore, it is essential to understand whether metal can kill, permanently damage or merely cause stress to the seagrass.

Morphological traits of seagrass, in particularly leaf dimension, 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 trait 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; Mal et al., 2002; Tan-Kristanto et al., 2003).

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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; Lajus and Zhang, 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). Thus, the main goal of this study was to observe the heavy metals effect on growth and leaf asymmetry of seagrass in a controlled environment.

MATERIALS AND METHODS

Plant material

Halophila ovalis was the seagrass species chosen for this study due to the ability of this species to grow faster than other seagrasses (Butler and Jernakoff, 1999), and its convenient size for the laboratory condition. *H. ovalis* were collected from uncontaminated area of Lake Macquarie (Fennel Bay; 151°66'E, 33°08'S) and transported to the laboratory free of sediment in the container filled with lake water, following McMillan (1980). The uncontaminated site were selected based on result from Ambo Rappe et al. (2007).

In the laboratory, the plants were sorted based on the size and the number of leaves. Individual with 3 pairs of mature leaves and 1 growing leaf were selected. The first pair of mature leaves was harvested and placed it in labeled jars for further analysis.The plants were grown in culture tubs (11.5 × 5 × 17 cm) filled with terrestrial sandy loam sediment. *In situ* sediment was not used as it may contain an unknown amount of contaminants, following Ralph and Burchett (1998).

Each culture tub contained 5 individual plants, which were arranged in the same direction of growth (that is, grown plant with older leaves was in the right section of the tub followed by the younger part to the left side). Twelve 15 L glass tanks were filled with filtered (200 µm) seawater and two culture tubs of *H. ovalis* were placed in each tank under 200 µmol quanta m^{-2} s^{-1} with a photoperiod of 16 h light and 8 h dark. The tank has a filter installed to aerate and filter the water during the entire experiment.

Heavy metal experimentation

A fully randomized experimental design with six treatments in two replicates totaling 12 tanks was used. The treatments were lead (10 and 50 mg/L), copper (0.5, 2, and 4 mg/L), and control (no addition of heavy metals). Lead and copper were selected for this experiment because these metals represent non-essential and essential metal, respectively, for seagrass growth. The metal concentrations were used in this study based on previous findings that exposure to copper concentrations more than 4 mg/L have a lethal effect on *H. ovalis*, whereas only limited effects of lead were shown to concentrations up to 10 mg/L (Ralph and Burchett, 1998).

Lead (Pb) and copper (Cu) solutions were produced by dissolving lead (II) nitrate (($Pb(NO_3)_2$) and copper chloride salt ($CuCl_2$), respectively, with distilled water. 10 000 mg/L Pb and 1000 mg/L Cu stock solutions were made. Since the volume of the tank was 15 L, addition of 15 and 75 ml of 10 000 mg/L Pb stock solution produced the treatment concentration of 10 and 50 mg/L Pb, respectively. Addition of 7.5, 30, and 60 ml of 10 000 mg/L Cu stock solution produced the treatment concentration of 0.5, 2, and 4 mg/L Cu, respectively. To remove any treatment effect caused by adding different amounts of stock solution, 60, 67.5, 45, 15, and 75 ml of distilled water was added to the 10 mg/L Pb treatment, 0.5 mg/L Cu treatment, 2 mg/L Cu treatment, 4 mg/L Cu treatment, and the control, respectively. Tanks were arranged in line in a random order to remove possible differences of environmental conditions in laboratory.

Water quality parameters such as salinity, temperature, pH, dissolved oxygen, and turbidity were measured weekly using Yeo-Kal (Model 611) water quality analyzer. Salinity was maintained by addition of tap water to compensate for losses due to evaporation, following McMillan and Moseley (1967) and McMillan (1976). The water quality parameters remained steady and constant between control and heavy metal dosed tanks with salinity values in the range of 33.88 to 34.08 ppt, temperature 26.65 to 26.79ºC, pH 8.00 to 8.03, DO 5.05 to 5.27 ppm and turbidity 0.56 to 0.89 ntu. Therefore, the water quality parameters among the tanks do not appear to have had an effect on the overall stress response in this study.

Growth measurement

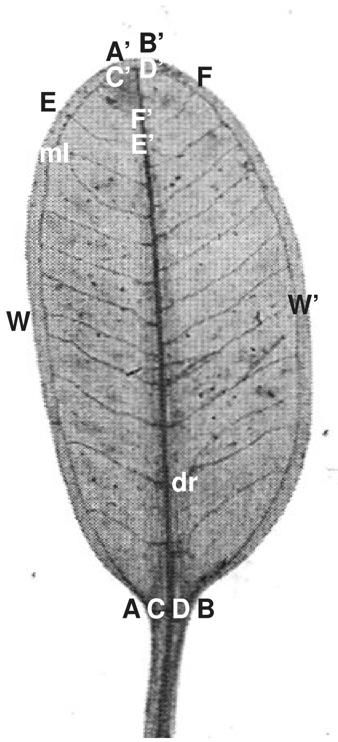
Images of the plants inside the tanks were taken using a digital camera at the beginning of experiment and then at 2, 5, 10, 18, 34, 42 and 51 days after the treatment. The measurements of the images were taken using 'Image Tool' software (developed at the University of Texas Health Science Centre at San Antonio, Texas). The overall growth of the *H. ovalis* during the experiment was estimated by counting the number of nodes and measuring the length of the rhizome. Growth rate per day was estimated from each treatment at two times, Time 0 day (T1) to time 18 days (T2) and time 18 days (T1) to time 51 days (T2), using the following formula:

(Length of the rhizome at T2 - Length of the rhizome at T1) / number of days

Fluctuating asymmetry (FA) and leaf dimension measurement

For the purpose of FA and leaf dimension data, a pair of leaves was harvested at the start of the experiment (before metals treatment), 18 days after the treatment, and 51 days (the end of the experiment). Time of harvesting was selected based on the information from the previous pilot study that *H. ovalis* produced a mature leaf at about 16 days in the tank. However, some of the plants in the copper treatments did not grow a mature leaf as quickly as other treatments; therefore, time 18 days was chosen to give sufficient time for these copper treatments. The final data of FA was taken at 51 days after observation that there was no further signs of new leaf growth, especially from copper treatment, thus the experiment was finalized.

Eight traits were analysed for leaf asymmetry of *H. ovalis* including 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), leaf perimeter (Trait 6), leaf length (Trait 7), and leaf width (Trait 8). Traits 1 to 5 were bilateral characters that measured leaf asymmetry within a leaf (Figure 1), whereas traits 6 to 8 was bilateral characters that measured leaf asymmetry between a pair of leaves. Length of veins was measured as sum of distances between intersections of lateral veins with peripheral or central



Trait 1, number of lateral vein intersections to the peripheral vein; A-A'(left) & B-B' (right)

Trait 2, number of lateral vein intersections to the central vein; C-C'(left) & D-D' (right)

Trait 3, length of peripheral vein: A-A' (left) & B-B' (right)

Trait 4, length of central vein: C-C' (left) & D-D' (right)

Trait 5, length of first lateral vein: E-E' (left) & F-F' (right)

Leaf-width (W-W') and leaf-length (D-D').
Additional points mark location of diverging or
merging of veins using symbol **ml** (merging on the left side), **dl** (diverging on the left side), **mr** (merging on the right side), **dr** (diverging on the right side).

Figure 1. Measurement of fluctuating asymmetry (Traits 1 to 5) on the leaf of *H. ovalis*.

veins. Leaf perimeter was measured as a sum of distances between intersections of lateral veins with peripheral veins on both the left and the right side of the leaf. The length of mid-vein was measured as a leaf length and the width of leaf was measured from one side of the leaf margin to the other side at its widest section.

The initial and repeat measurements of each trait were evaluated by conducting two-way mixed ANOVA with side and individual as random factors. The test revealed that measurement error was responsible for 17.03, 17.28, 9.32, 168.08, 16.74, 11.77, 8.48 and 15.63% of traits 1 to 8, respectively. Due to the high measurement error on trait 4, this trait was excluded from further analyses. Other traits have low measurement error (< 20%) and could thus be used for the next step of analysis.

The two-way ANOVA also revealed the non-significant difference in factor "Side" in traits 1, 2, 3 and trait 8, indicating absence of directional asymmetry (DA). Other traits (Traits 4 to 7) showed directional asymmetry. Palmer and Strobeck (2003) suggested avoiding using traits that exhibit DA because it would complicate the FA analyses. Therefore, traits 5 to 7 were also excluded from further FA analysis.

There was also a significance of the interaction factors "Individual x Side" for all traits indicating the presence of non-directional asymmetry, which may relate to FA. However, it is important to know if another type of non-directional asymmetry, antisymmetry, contributes in the non-directional asymmetry observed in this data. To test for antisymmetry, the distribution of left minus right within each trait selected was observed visually and checked for the departure from normality using Kolmogorov-Smirnov test (Sokal and Rohlf, 1995). The data was also checked for statistical outlier that is a common source of skew or leptokurtosis in studies of fluctuating asymmetry (Palmer, 1994).

Kolmogorov-Smirnov test revealed that (L-R) distributions of trait 8 did not deviate from the normal distribution, but the distribution deviated from zero for traits 1 to 3. Significant positive kurtosis was detected for (L-R) distribution of traits 1 to 3 (p<0.001) indicating leptokurtic distribution (Sokal and Rohlf, 1995). Leptokurtosis can be caused by number of reasons such as outliers, measurement errors, and the mixture of ideal FA and antisymmetry (Palmer and Strobeck, 2003). The other two reasons (outliers and measurement errors) were controlled in this study, however no methods have yet been suggested to statistically correct for the presence of antisymmetry, therefore care should be taken to show that inferred differences in developmental stability among samples are not confounded by this factor (Palmer, 1994).

From the two-way ANOVA test, significant variation was also found dealing with the overall trait size or shape among individuals. A dependence of asymmetry on trait size can influence inferences made in studies of developmental stability, therefore, a size-correction was conducted by standardizing the asymmetry data with leaf length. The FA index was then calculated based on unsigned left minus right characters |L - R| / (R + L) (Palmer, 1994).

Statistical analysis

Analysis of variance (ANOVA) (GMAV 5, University of Sydney 2000) was conducted to compare all the variables measured between metal treatment (Pb and Cu) and control.

The length of plant and number of nodes were analyzed using 2 fixed orthogonal factors ANOVA: Time (8 levels) and treatment (6 levels), with n = 20 per combination of factors.

FA of bilateral characters within a leaf (Traits 1, 2, and 3) and leaf dimension characters (leaf length and leaf width) were first analyzed prior to the metal treatment (Time 0 day) using four factors ANOVA: Treatment (6 levels, fixed orthogonal), tank (2 levels, nested in treatment), tub (2 levels, nested in tank), plant (5

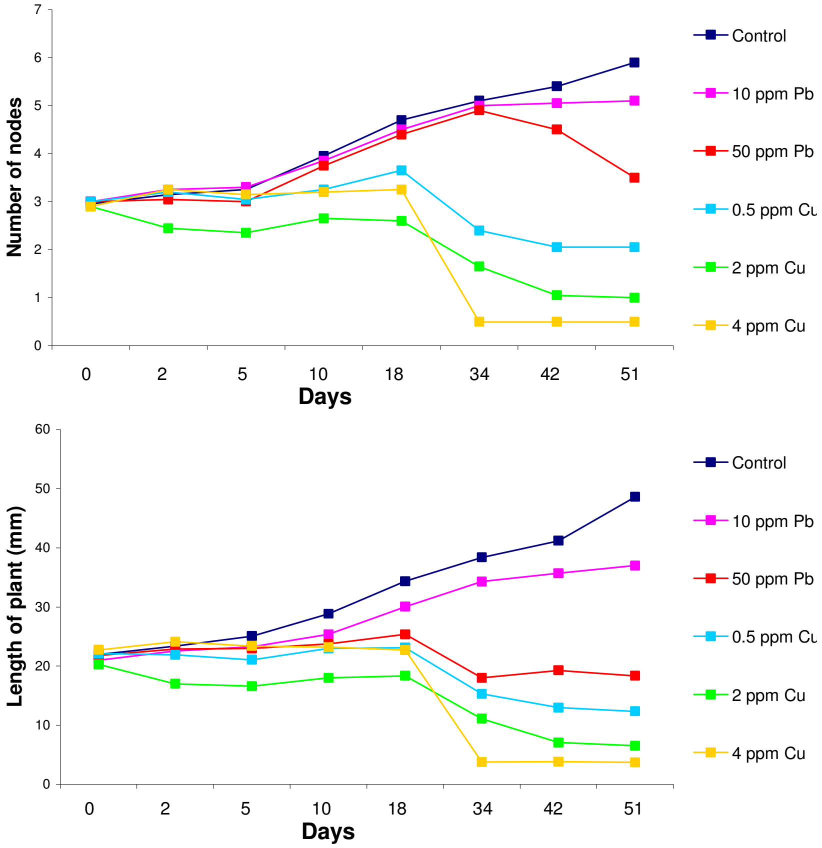


Figure 2. Mean number of nodes (*n* = 20) and length of plants (*n* = 20) at different metals treatments and at different duration of the experiment

levels, nested in tub), with *n* = 2 per combination of factors. Whereas, FA of bilateral character within the plant (Trait 8) was analyzed at time-0 day using three factors ANOVA: Treatment (6 levels, fixed orthogonal), tank (2 levels, nested in treatment), tub (2 levels, nested in tank), with *n* = 5 per combination of factors. These analyses were performed to see whether there were other factors rather than metal treatment confounding the result.

For the final result, data on FA and leaf dimension characters were pooled from individual plants, tubs, and tanks due to the limited number of data available, especially from the metal treatments. Therefore, analysis was only performed on the more important factors, in this case, treatment and time. Thus, two fixed factors ANOVA: Time (3 levels) and treatment (6 levels) was used.

Prior to the analysis of variance, all data were tested for heterogeneity of variance using Cochran's test. When the Cochran's test was significant, the data were transformed. For all ANOVA analyses, significance was determined at the α = 0.05. Moreover, Student-Newman-Keuls (SNK) tests were used if a significant effect was found to determine which level of the factor differed significantly.

RESULTS

Growth responses

Growth rate of *H. ovalis* was influenced by heavy metals (lead and copper) ($F_{5.114}$ = 10.17, p<0.001) and SNK tests indicated a difference between control and all metal treatments, with exception for 10 mg/L Pb. Plants in the control treatment grew constantly at a rate of 0.69 mm/day on the first 18 days and continued to grow at a slower rate (0.43 mm/day) during the period of 18 to 51 days. Although plants in the 10 mg/L Pb had a similar pattern of growth to the control, but the growth was lower (0.5 mm/day on the first 18 days and 0.21 mm/day at 18 to 51 days). Plants exposed to 50 mg/L Pb had a slower growth rate than plants in 10 mg/L Pb (p<0.05) and the difference with control was even greater (p<0.01).

The growth of the plant in the 50 mg/L lead treatment was only observed for up to 18 days of the experiment (Figure 2). Plants treated with the relatively low concentrations of Cu (0.5 mg/L) showed a significantly slower growth rate than the control (p<0.01), and the growth was also observed for up to 18 days only. The toxic effects of copper on the growth of *H. ovalis* were more apparent at the higher concentrations (2 to 4 mg/L), since increasing inhibition of rhizome elongation was observed with increasing Cu concentration (Figure 2). The rhizomes of the plants (measured for this parameter) were still intact but some appeared decayed over the time. Therefore, the measured length was also becoming smaller.

Table 1. Summary of ANOVA for leaf length, leaf width, leaf width to length ratio of *H. ovalis* after the metal treatments.

Source of variation	df	Leaf length (LL)		Leaf width (LW)		Ratio LW/LL	
		MS	F	MS	F	MS	F
Time	2	64.03	244.83 ***	14.23	250.02 ***	0.004	17.86 ***
Treatment	5	5.80	22.19 ***	0.76	13.3 ***	0.002	8.55 ***
Time × Treatment	10	2.29	8.75 ***	0.43	7.57 ***	0.001	4.56 ***
Residual	18	0.26		0.06		0.000	

Cochran's tests were not significant, data were not transformed.*** denoted significant at $p<0.001$.df, degrees of freedom, MS, mean square, F, Fisher-test.

There was a positive correlation between length of plants (measured from the length of rhizome) with the number of nodes (Spearman correlation, $p<0.001$). The plants become longer as the number of nodes increases (Figure 2). In relation to the inhibition of growth, the length of the plant (measured from the length of rhizome) and the number of nodes were also influenced by the heavy metals ($F_{5.912} = 40.99$, $p<0.001$ and $F_{5.912} = 58.54$, $p<0.001$, respectively). SNK tests indicated a difference between control and all metal treatments. The plants exposed to 50 mg/L Pb and to different level of copper treatments were shorter with fewer nodes than control ($p<0.01$). The plants exposed to 10 mg/L Pb were shorter compared to the control ($p<0.05$), but there was no difference to control in the number of nodes.

The duration of metal exposure influenced the length of the plants and the node number ($F_{7.912} = 2.04$, $p<0.05$ and $F_{7.912} = 4.15$, $p<0.001$). Moreover, the significant effect of the interaction between the metal treatment and the metal exposure indicated that exposure time also influenced the effects of the metals in determining the plant characters ($F_{35.912} = 5.23$, $p<0.05$ for length of plant and $F_{35,912} = 7.33$, $p<0.001$ for number of nodes). SNK test revealed a difference between 0 day and 34 to 51 days of the metal exposure. Plants exposed to 10 mg/L Pb show a decrease in length and number of nodes after 51 days of exposure, whereas plants exposed to higher Pb concentration (50 mg/l Pb) show a decrease in length after 34 days of exposure and a decrease in number of nodes after 42 days of exposure. Plants treated with different levels of copper decreased the length and number of nodes after 34 days of copper exposure (Figure 2).

Leaf dimension characters

The initial leaf length and leaf width, before adding metal treatments, did not differ among control and the various treatment levels. There was also no difference in these parameters among the tubs used as a media culture. The only difference was observed among the individual plants within the tub, but this factor was pooled in the final analysis. After the treatment, leaf dimension characters (e.g. leaf length, leaf width and leaf width to length ratio) were significantly different between treatments and control and also between times (Table 1).

In this analysis, data were pooled from tubs and individual plants due to the limited number of leaves which survived. The mortality of leaves was especially high in copper treatments where more than 50% of the leaves became senescent and detached from the rhizome only after 2 days of the copper treatment. SNK tests indicated a difference between the control and copper treatments, but no such difference was observed between control and lead treatments. Plants exposed to lead had a similar leaf length and width to control, but plants treated with copper had a smaller leaves than the control (Figure 3). SNK tests also indicated an effect of the metal treatment when comparing leaf length and width in two intervals: From the beginning of the experiment up to 18 days after treatment, and for days 18 to 51. There was a general pattern across the treatments that new leaves growing in the tank were smaller in size (in term of reduced leaf length and width) with time. This general pattern may be due to the effect of the culture tank that had no nutrition addition during the experimentation. This phenomenon was also previously observed in the pilot study.

The interaction of metal treatment and exposure time of the treatment was also analyzed using SNK tests and the tests indicated plants in control and lead treatments only reduced their leaf length and width at 18 days and the leaves that grew after this period had the same or even larger size. Plants exposed to copper treatments, however, continued to have smaller leaves throughout the time with the effect becoming more apparent at the higher Cu concentrations (Figure 3).

High metal concentrations (50 mg/L Pb and 2 to 4 mg/L Cu) caused also change of shape of leaves measured as width to length ratio as indicated by SNK tests. Plants exposed to these higher metal concentrations had larger width to length ratio (that is, wider leaves) than control. The SNK tests also revealed a difference in width to length ratio of the plant between 0 and 51 days, but the pattern was not consistent among the treatments.

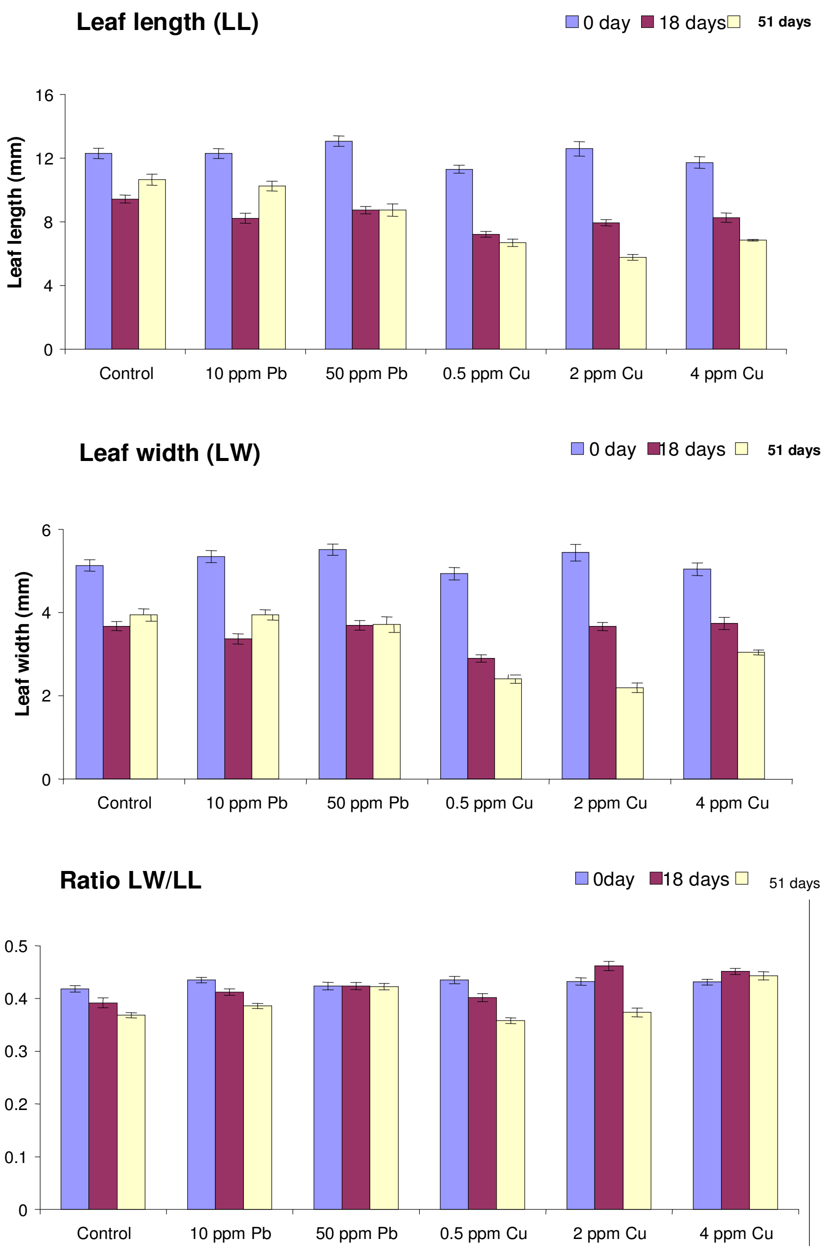


Figure 3. Mean (±SE, *n* = 40) length, width, and width to length ratio of *H. ovalis* leaves at different treatment levels and at different duration of the experiment.

Fluctuating asymmetry (FA)

The fluctuating asymmetry characters (Traits 1, 2, 3 and 8) before adding the metals did not differ among the control and different treatments. There was also no difference in fluctuating asymmetry between leaves from different tanks and from different individual plants. After the treatment, however, there was an increased fluctuating asymmetry (showing from all traits measured) between treatments (Table 2) with SNK tests indicating a difference between control and 2 mg/L Cu, which exhibited an increased FA. Although other metals treatments show a higher FA than control, the differences were not significant (Figure 4).

The difference in time exposure of the metals on the leaf asymmetry was significant for traits 1 and 2 (Table 2) with SNK tests indicated a significant increased in leaf asymmetry after 51 days of the metal exposure. Traits 1 and 2, which are meristic characters dealing with number of veins, show a similar effect in relation to the treatment. These traits were correlated each other with the coefficient of correlation (*r* = 0.83). These traits are in concordance, thus using one of the traits will be able to reflect the FA in the same way as using both traits. These

Table 2. Summary of ANOVA for FA characters of *H. ovalis* leaf after the metal treatments.

Source of variation	df	Trait 1		Trait 2	
		MS	F	MS	F
Time	2	0.51	5.64 *	0.46	5.21 *
Treatment	5	0.34	3.76 *	0.38	4.33 **
Time × Treatment	10	0.19	2.13 ns	0.12	1.39 ns
Residual	18	0.09		0.09	

Source of variation	df	Trait 3		Trait 8	
		MS	F	MS	F
Time	2	0.00	1.39 *ns*	0.00	0.93 *ns*
Treatment	5	0.00	4.94 **	0.00	4.88 **
Time × Treatment	10	0.00	2.79 *	0.00	2.24 *ns*
Residual	18	0.00		0.00	

Traits 3 and 8 were not transformed (Cochran's test >0.05), Ln(X) transformation for traits 1 and 2. *ns* - not significant, * - significant at $p<0.05$, ** - significant at $p<0.01$. df, degrees of freedom, MS, mean square, F, Fisher-test.

traits consistently showed a significant increase in leaf asymmetry of plants exposed to 2 mg/L Cu compared to control. Moreover, the leaf asymmetry significantly increased in all copper treatments after 51 days of copper exposure. Although plants exposed to 50 mg/L Pb also show an increased FA after 51 days of the treatment, the pattern was not significant (Figure 4).

Fluctuating asymmetry of the length of peripheral vein (Trait 3), on the other hand, showed more complicated pattern and this trait only indicated the significant increase in leaf asymmetry of the plants treated with 2 mg/L of copper. Similar to traits 1 and 2, this trait also exhibited significantly higher FA than control after 51 days of the copper exposure.

Trait 8, which comparing the width of a pair of leaves, has greater FA in high copper treatments (2 to 4 mg/L) than control, but the difference was significant in plants exposed to 2 mg/L Cu only (Figure 4).

DISCUSSION

Lead and copper inhibited the growth of seagrass, *H. ovalis.* The effect was more pronounced at higher concentration of heavy metals and at longer exposure. For example, 0.5 mg/L Cu had reduced growth of *H. ovalis* in comparison with control, but the plant still grew very slowly up to 18 days, and no growth was observed after this period. Moreover, the plants exposed to 2 to 4 mg/L Cu had progressively reduced the extension of rhizome resulting in shorter plants with a reduced number of nodes. In that higher copper treatment, the plant rhizome became decayed shortly after the treatment. Thus, there was no growth observed in the plants treated with those higher copper concentrations.

Copper, although an essential nutrient for plants, when absorbed in excess amounts can cause deleterious effects at morphological, physiological and ultrastructural levels (Ouzounidou et al., 1992). Specifically, excess copper can cause chlorosis, inhibition of root growth and damage to plasma membrane permeability, leading to ion leakage (De Vos et al., 1991; Ouzounidou et al., 1992). A reduction of root extension at the concentration of 30 to 160 µM of copper have also been demonstrated in a terrestrial plant *Alyssum montanum*, where Cu^{2+} attack sulphydryl groups causing damage to permeable layers and allowing the diffusion of ions into the chloroplast (Ouzounidou, 1994).

Leaf senescence was also occurred in *H. ovalis* 2 days after exposing the plant to copper treatments, with more than 50% of leaves prematurely senesced and detached from the rhizome after exposure to the higher copper concentrations (2 to 4 mg/L). The effect of copper exposure on leaf senescence found in this study was consistent with previous studies (Ralph and Burchett, 1998; Prange and Dennison, 2000). This phenomenon is suggested to be associated with the stimulation of phytochrome activity, leading to increased abscisic acid and ethylene production, compounds that are precursors of leaf abscission and loosening of cell walls (Malea, 1994).

Similar to copper, lead also reduced the *H. ovalis* growth rate and the effect was more apparent at the greater concentration and longer exposure period. Thus, 10 mg/L Pb had reduced the growth rate of the plant to about 28% up to 18 days and the growth rate reduced to 51% thereafter until the end of the experiment (a further 33 days). The more extreme effect was observed at higher concentrations (50 mg/L), where the plants had reduced growth rate of about 71% up to 18 days, and no growth was observed after this period. Comparing with the copper effect, however, the effect of lead on *H. ovalis* growth was weaker. Lead (a non-essential element) displayed only limited effect on the growth of *H. ovalis*,

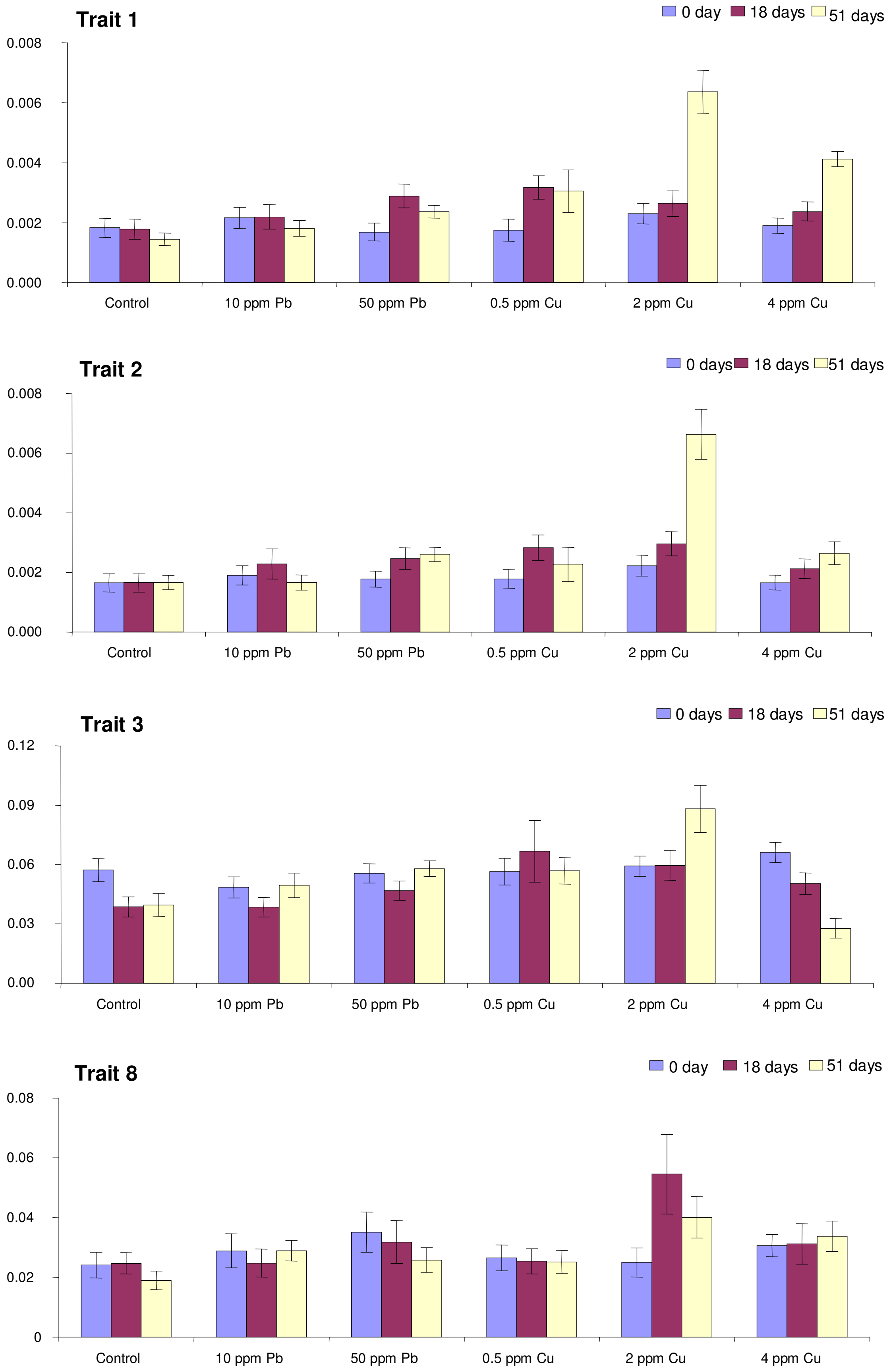


Figure 4. FA of *H. ovalis* leaves at different treatment levels and at different duration of the experiment.

whereas copper (an essential element), even at lower concentrations, had significantly greater effect. The evident negative effect of essential micronutrient heavy metal on growth could be associated with uptake and exclusion mechanisms.

Since copper is required for metabolic processes, the plant actively takes up copper; however, when exposed to increased concentrations, uptake may exceed metabolic requirements and result in a toxic impact. On the other hand, lead is not required for growth or development; the plant may actively exclude this element to minimize the toxic impact by isolating this element in storage tissues, where it does not interfere with the metabolic activity. Ward (1989) postulated that seagrasses tolerate high concentrations of heavy metals by sequestering them into structural components of the leaf tissue, therefore preventing them from affecting the more sensitive metabolic processes. Similar mechanisms have been reported in algae (Pinto et al., 2003). Moreover, MacFarlane and Burchett (2000) reported the limited translocation of Pb in the grey mangrove *Avicennia marina*. They found Pb concentrated in root tissue, with only minimal transport to the shoot. This evidence also suggested that Pb has a limited effect on plants growth and development due to the limited translocation of this element through the vascular system to the leaves/shoot, where the metabolic activity is mostly occurred.

The results of the effect of Cu and Pb on the growth of the seagrass, *H. ovalis*, presented in this study are in good agreement with other studies. For example, it has been reported that 1 mg/L copper had an effect on photosynthetic activity of *H. ovalis*, and the concentrations of 5 to 10 mg/L Cu had a lethal effect on this plant, whereas concentrations of 1 to 10 mg/L Pb had only limited effect (Ralph and Burchett, 1998). It has also been suggested that Cu can be toxic to microalgae at concentrations of 0.19 to 0.3 µg/L, which is only marginally above those found in oceanic waters, while Pb reduces growth of this microscopic plant at concentration above 20 µg/L (Langston, 1990). Lead treatment did not significantly affects the leaf dimension characters, but copper treatment did affect these characters by a decrease in leaf size (measured by leaf length and width) and the effect became more apparent as the copper concentrations increased and also with a longer time exposure.

On the other hand, width to length ratio of the *H. ovalis* leaves was greater in higher metal levels for both lead and copper. Reduced leaf size and an increased width to length ratio may be a mechanism of the plant to survive under environmental pressures, in this case under heavy metal stress. It was suggested that toxic concentrations of heavy metals inhibit metabolic activity, and plants that survive do so with decrease growth and slower development (Clijsters and Van Assche, 1985). Seagrass, *Zostera capricorni*, from unpolluted locations decreased leaf length when growing in polluted sediment (Conroy et al., 1991). In contrast, it was found from the field study that seagrass, *H. ovalis*, grew in the metal polluted location had considerably longer and wider leaves compared to the ones in unpolluted locations suggesting the effects of uncontrolled factors such as nutrients, which may potentially compensate or even surpass the effect of heavy metals on the seagrass (Ambo Rappe et al., 2008).

The present experiment also demonstrates that heavy metals stress could increase FA in *H. ovalis.* FA as an ecological indicator may thus have potential as sensitive tools for monitoring the quality of the environment (Tracy et al., 1995; Anne et al., 1998; Hoffmann and Woods, 2003; Lajus and Zhang, 2003; Leung et al., 2003). Reduced growth rate and also leaf size of *H. ovalis* have been documented, especially with increased copper treatment and increasing of the duration of exposure to the metal. Increased of FA at this metal treatment was observed as well. The experiment thereby succeeded in discriminating between non-stressful and stressful conditions due to heavy metals. Meristic traits (Traits 1 and 2) dealing with the number of veins seems to be suitable, especially for leaf asymmetry with a bilateral character. Metric traits (Traits 3 and 8) dealing with the perimeter of the leaf is also a potential trait to be used, even though they still need to be examined further due to the inconsistency of the results on some occasions. It has been suggested that 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 et al., 2005). Different characters may also exhibit different patterns of fluctuating asymmetry depending on their size (Lajus, 2001). Selecting 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). Meristic traits that have proven to have a good sensitivity to heavy metal stresses in *H. ovalis*, is cross veins that join and interconnect a midvein with two lateral ‘intra-marginal veins’. These branched cross veins is positioned on either side of the midrib (Kuo and den Hartog, 2001; Coles et al., 2004; Kuo and den Hartog, 2006).

Like other veins, these branched cross veins have an important function in the vascular system of plants, which is related to the transport of solution within the plant (Kuo and den Hartog, 2006). It might be possible that the heavy metals pressure causes the imbalance of development of this structure on the right and the left side of the midrib that is normally in balance number (Freeman et al., 1993), leading to increased developmental instability (measured as FA). Plants exposed to 2 mg/L Cu exhibited significantly higher leaf asymmetry, while no significant leaf asymmetries were observed in plants treated with lead or with low levels of

Cu (0.5 mg/L).

Conclusion

This experimental study showed that copper (Cu) could lead to reduced growth rate and increased leaf asymmetry in *H. ovalis*. Leaf size of the plant also reduced as Cu level increased and when the plants were exposed to the heavy metal for longer duration. Copper, being an essential metal, resulted in a more pronounced effects on the plant characteristics than lead (non-essential metal), especially at higher concentrations. Thus, the experimental data showed quite a notable effect of heavy metals on various characteristics of seagrass, *H. ovalis*. It is, therefore, important to emphasize that the effect is observed at concentrations, which do occur in the wild and thus can be used for interpretation of the field data.

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