Bull. Environ. Contam. Toxicol. (2003) 70:90–96

DOI: 10.1007/s00128-002-0160-0

Bulletin of Environmental Contamination and Toxicology

Chlorophyll *a* Fluorescence as a Potential Biomarker of Zinc Stress in the Grey Mangrove, *Avicennia marina* (Forsk.) Vierh.

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Received: 12 March 2002/Accepted: 18 August 2002

Heavy metals often occur in urban estuarine ecosystems due to their proximity to industrial activity, urban runoff and boating activities. Zinc often occurs in high concentrations in polluted estuarine sediments, up to 800 -1000 μg/g (Luoma, 1990). Mangroves are important primary producers in estuarine systems, and appear to possess a tolerance to relatively high levels of heavy metals. A number of researchers have found high concentrations of accumulated metals in the tissues of a variety of mangrove species in both the field & laboratory including, *Avicennia* spp., *Kandelia* spp. and *Rhizophora* spp. (de Lacerda, 1998; Peters *et al.* 1997). Although considered tolerant, uptake of essential metals in excess to nutritional requirements by mangroves may induce a number of subcellular responses, that is, metabolic reactions, which can cause damage at the cellular level, or possibly lead to wider phytotoxic effects (Vangronsveld & Clijsters 1994).

Inhibition of photosynthesis by heavy metals in higher plants is well documented (Clijsters & Van Assche 1985, Prasad & Strzalka 1999), especially in those which utilise a C_3 photosynthetic chemistry, including *A. marina* (Ball 1985, Basak *et al.* 1996). Chlorophyll *a* fluorescence is a well established technique for the assessment of photosynthetic rate and plant stress (Judy *et al.*, 1990; Krause & Weiss, 1991) and has been used in a number of studies as a diagnostic tool for sub-lethal stress in response to heavy metal exposure in terrestrial angiosperms (Lanaras *et al.*, 1993; Maksymiec *et al.*, 1994) and aquatic plants (Ralph & Burchett, 1998). Chlorophyll *a* fluorescence has been explored in mangrove species in response to a number of environmental variables (Ball & Critchley, 1982; Ball, 1985; Cheeseman *et al.*, 1997; Sobrado, 1999), yet no studies todate have explored the effects of metals on the photosynthetic efficiency of mangroves using chlorophyll *a* fluorescence.

Monitoring sub-lethal changes in photosynthesis, via a technique such as fluorescence, in response to metal stress not only indicates damage to the photosynthetic apparatus and accompanying changes in photosynthetic capacity, but also has consequences for reduced carbon assimilation, growth, survival and reproduction (Vangronsveld & Clijsters 1994). Such effects may have implications for mangrove individuals and populations in the long term.

Thus the aim of the current study has been to investigate the effects of Zn exposure on chlorophyll *a* fluorescence in the grey mangrove, *Avicennia marina* (Forsk.) Vierh. under laboratory conditions.

MATERIALS AND METHODS

Mature *A. marina* propagules were collected from Powells Creek, Homebush Bay, Sydney, Australia (33° 06'S, 151° 09'E). Only complete, undamaged propagules with testa intact and no emergent hypocotyl or radicle were selected for planting. The propagules ranged between 10- 10.4g fresh weight. Propagules were grown for 6 months under glasshouse conditions in a commercially prepared soil (50% silty clay loam, 20% washed river sand, 30 % organic peat moss) in 20% seawater with the fertilizer Aquasol (0.8 % w/v), in order to mimic an estuarine sediment representative of the region. The seedlings were planted in separate 140 x 140 mm plastic pots immersed within 2L plastic containers (as holding trays) to minimise drainage and simulate anoxic, waterlogged conditions. Seedlings were watered manually. Levels of water in the holding trays were maintained at 300mL and re-percolation was carried out once a week.

Thirty, six month old seedlings were chosen for experimentation. All seedlings were similar in apparent health, height (318 ± 29 mm), and leaf number (8 ± 0.86) (mean ± SE). Seedlings were randomly allocated to each treatment (n = 6). To each individual pot in each treatment, an appropriate solution of the Zn metal salt was added to arrive at 6 replicates across the concentration range, 0, 125, 250 ,500 and 1000 μg Zn (as $ZnCl_2$) per gram of dry sediment. Zinc was added as the chloride salt to minimize adverse effects of anion toxicity, mangroves being adapted to high chloride levels in estuarine sediments (MacFarlane & Burchett, 2000). Dosed replicates were block randomised and maintained in the glasshouse for a period of 8 weeks prior to harvest.

Fluorescence measurements were determined with the PAM-2000 Fluorometer (Walz, Germany). Data analysis was performed using the DA-2000 software version 2.00 (Walz, Germany). The background chlorophyll *a* fluorescence signal (F_o) was excited by continuous 3μs pulses of red measuring light (655nm, 0.3 μmol $m^{-2}s^{-1}$) applied at a frequency of 600Hz. Induction of the maximum fluorescence (F_m) was achieved with a short pulse of white 'saturating light' (4000 μmol $m^{-2}s^{-1}$). The derived parameter, PSII photochemical efficiency, the F_v/F_m ratio (where $F_v = F_m - F_o$), was also calculated. All fluorescence signals were measured by a photo-diode at wavelengths of higher than 700nm. Fluorescence measurements were performed at harvest (eight weeks), between 7:00 and 8:00 hr to minimise the effect of diurnal fluctuations in photosynthetic activity (Hanelt *et al.*, 1993). Measurements were performed on each seedling from each treatment, one reading obtained from each of the second fully expanded intact leaves, and values averaged per plant. Leaves were washed with MilliQ water prior to measurement to remove accumulation of dust and excreted salts. Dark adaptation clips (Walz, Germany) were used to dark adapt the leaves and ensure that the distance between the fibre optic head and leaf sample was

constant. The fluorescence signal was measured at a standard position, in the middle of the leaf, avoiding the mid-vein, on the adaxial surface. The dark adaptation period was 20 minutes. Preliminary investigations suggested a dark adaptation period of 20 minutes was reproducible and not significantly different from a 40 minute period.

After harvest, 500 mg samples (dry weight) of washed, oven dried and homogenized second leaf pairs, or sediment (n=6), were weighed separately into acid washed (10% HNO3) beakers (100mL) and digested for Zn analysis with a hot mixture of concentrated nitric acid and hydrogen peroxide, after the method of Krishnamurty *et al.* (1976). Analysis carried out on the resultant digestions using atomic absorption spectroscopy (air/acetylene, Varian AA-1275, Australia). Standards used were matrix matched and international plant/ sediment standards (NBS standard reference materials; 1646/estuarine sediment; 1572/citrus leaves) were used to check percentage recovery of metals (Sediment Zn = 84%; Leaf tissue Zn = 94%).

Differences among treatments were assessed with a one factor analysis of variance (ANOVA) in STATISTICA (StatSoft, 1995). Relationships were examined using linear bivariate regression analyses in SigmaPlot (1997).

RESULTS AND DISCUSSION

Within one week of Zn addition, acute toxicity was observed only in the 1000μg/g treatment with seedlings displaying severe stress symptoms, including chlorosis. Plants in this treatment were harvested one week after initial metal exposure. Seedlings exposed to 125μg/g Zn and above were found to accumulate significantly more Zn in leaf tissues than the control treatment (Table 1). The accumulation of Zn in leaf tissue was found to be proportional to sediment Zn levels (r^2= 0.86, p<0.01). *A. marina* may be classified as an indicator species for Zn accumulation (Baker & Walker 1990).

The accumulated Zn levels in leaf tissues were on average 3 times lower than sediment metal concentrations, suggesting an exclusion mechanism to accumulation. *A. marina* can sequester much of the accumulated Zn root tissue with limited translocation to leaf tissue. Zn, although mobile, shows restricted translocation to the shoot due to the endodermal casparian strip (MacFarlane & Burchett, 2000). Exposure to Zn resulted in changes in photosystem II (PSII) photochemical efficiency (Table 1). A significant decline in the F_v/F_m ratio was observed in the 500μg/g Zn treatment, i.e. concentrations lower than those which induced visible signs of toxicity. Changes in PSII photochemical efficiency can be attributed to significant declines in maximum fluorescence, F_m, at concentrations of 500μg/g Zn. The minimum fluorescence, F_o, remained constant across the concentration range applied. Decreases in F_m, while F_o remains constant, suggests a Zn mediated alteration in thylakoid membrane structure, impacting on the electron transport rate, corresponding with an increase in energy dissipation. The accompanying reductions in PS II photochemical efficiency may also be a

consequence of Zn initiated damage to PSII photochemistry (de Filippis & Pallaghy, 1994; Ralph & Burchett, 1998).

Table 1. Sediment metals, fluorescence measurements (relative units) and accumulated metals in leaf tissue of *A. marina* after an 8-week exposure period to Zn.

Nominal sediment metal conc.	Minimum fluorescence (F_o)	PSII photochemical efficiency (F_v/F_m)	Maximum fluorescence (F_m)	Sediment Zn (μg/g)	Leaf Zn (μg/g)
0 μg/g Zn control	0.254 (0.037)	0.758 (0.083)*a*	1.125 (0.263)*a*	31.4 (1.3)*a*	26.8 (2.4)
125 μg/g Zn	0.284 (0.057)	0.737 (0.096)*ab*	1.121 (0.155)*ab*	158.0 (4.7)*ab*	54.1 (4.4)*a*
250 μg/g Zn	0.306 (0.067)	0.672 (0.120)*ab*	0.994 (0.212)*ab*	224.4 (13.2)*b*	76.9 (4.9)*ab*
500 μg/g Zn	0.286 (0.071)	0.653 (0.103)*b*	0.883 (0.266)*b*	452.6 (13.4)	95.6 (11.9)*b*
1000 μg/g Zn				898.1 (81.7)	245.2 (48.6)
ANOVA F	1.55 n.s.	2.92*	3.08*	123.1**	44.1**

Mean values ± (standard error), n = 6. Results of a one way ANOVA, ** = significant difference at $p < 0.01$, * = significant difference at $p < 0.05$. Treatments identified as similar, according to Tukey's HSD multiple comparison, are linked by identical letters

Accumulated Zn in leaf tissue also exhibited a linear dose response relationship with PSII photochemical efficiency, as Zn in leaf tissue increases, PSII photochemical efficiency decreases at sub-lethal Zn concentrations (Figure 1). Similarly, levels of chlorophyll *a* and chlorophyll *b*, and to a lesser degree carotenoids, have been found to decrease in a dose-dependant fashion with increasing exposure levels of Zn in *A. marina* leaf tissue (MacFarlane & Burchett, 2001). Decreases in pigments suggest that the chlorophyll synthesising system and chlorophyllase activity were affected at higher exposure concentrations (Van Assche & Clijsters 1990), while iron depletion, or substitution of magnesium with Zn may also be a contributing damage mechanism. Changes in membrane permeability and chloroplast ultrastructure may also contribute to declines in pigment levels due to lipid peroxidation (Prasad & Strzalka 1999).

Decreases in photosynthetic activity, implies reduced carbon fixation and possible effects at the whole plant level (Baker & Walker 1990). Furthermore, these impacts are seen at Zn concentrations lower than those required to induce visible phyto-toxicity. The significant linear dose –response relationship between accumulated Zn and PS II photochemical efficiency also suggests that chlorophyll *a* fluorescence is an appropriate biomarker for Zn stress in *A. marina*.

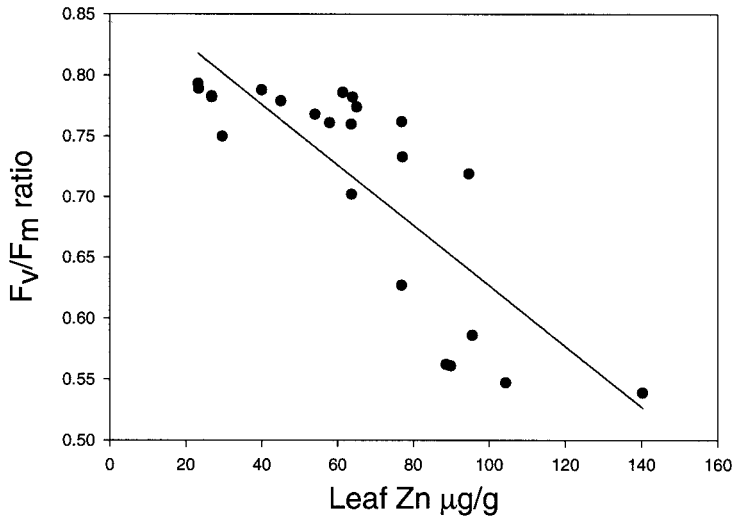

Figure 1. Relationship between accumulated Zn in leaf tissue and PSII photochemical efficiency (F_v/F_m ratio, relative units) in A. marina after 8 weeks exposure to sediment Zn ($r^2 = 0.65$, $p<0.01$).

Acknowledgments. I thank Peter Ralph, Cate Macinnis-Ng, Meg Burchett and Alex Pulkownik for assistance and advice, and funding support from Olympic Co-ordination Authority, Bi-centennial Park Authority and the University of Technology, Sydney.

REFERENCES

Baker AJ, Walker PI (1990) Ecophysiology of metal uptake by tolerant plants. In: Shaw AJ (ed) Heavy metal tolerance in plants; evolutionary aspects. CRC Press Inc, Boca Raton, Florida, pp. 155-178

Ball MC (1985) Photosynthesis in mangroves. Wetlands (Aust) 6: 12-22

Ball MC, Critchley C (1982) Photosynthetic responses to irradiance by the Grey mangrove, *Avicennia marina*, grown under different light regimes. Plant Physiol 70: 1101-1106

Basak UC, Das AB, Das P (1996) Chlorophylls, carotenoids, proteins and secondary metabolites in leaves of 14 species of mangrove. Bull Mar Sci 58: 654-659

Cheeseman JM, Herendeen LB, Cheeseman AT, Clough BF (1997) Photosynthesis and photoprotection in mangroves under field conditions. Plant Cell Environ 20: 579-588

Clijsters H, Van Assche F (1985) Inhibition of photosynthesis by heavy metals. Photosynth Res 7: 31-40

de Filippis LF, Pallaghy CK (1994) Heavy metals: sources and biological effects. In: Rai LC, Caur JP, Soeder CJ (eds) Algae and water pollution: advances in limnology series, Vol. 42. Schweizerbart, Stuttgart, pp 32-77

de Lacerda LD (1998) Trace metals in mangrove plants: why such low concentrations ? In: de Lacerda LD, Diop HS (eds) Mangrove ecosystem studies in Latin America and Africa. UNESCO, pp 171-78

Hanelt D, Huppertz K, Nultsch W (1993) Daily course of photosynthesis and photoinhibition in marine macroalgae investigated in the laboratory and field. Mar Ecol Prog Ser 97: 31-37

Judy BM, Lower WR, Miles CD, Thomas MW, Krause GF (1990) Chlorophyll fluorescence of a higher plant as an assay for toxicity assessment of soil and water. In: Wang W, Gorsuch J, Lower W (ed) Plants for toxicity assessment. ASTM STP 1091, Philadelphia, pp 308-318

Krause GH, Weiss E (1991) Chlorophyll fluorescence and photosynthesis: the basics. Ann Rev Plant Physiol 42: 313-349

Krishnamurty KV, Shpirt E, Reddy M (1976) Trace metal extraction of soils and sediments by nitric acid- hydrogen peroxide. Atom Absorpt Newsl 15: 68-71

Lanaras T, Moustakas L, Symeonidis L, Diamantoglou S, Karataglis S (1993) Plant metal content, growth responses, and some photosynthetic measurements on field-cultivated wheat growing on ore bodies enriched in Cu. Physiol Plant 88: 307-314

Luoma SN (1990) Processes affecting metal concentrations in estuarine and coastal marine sediments. In: Furness RW, Rainbow PS(eds) Heavy Metals in the Marine Environment. CRC Press, Boca Raton, Florida, pp 51-66

MacFarlane GR, Burchett MD (2000) Cellular distribution of Cu, Pb and Zn in the grey mangrove, *Avicennia marina* (Forsk.) Vierh. Aquat Bot 68: 45-59

MacFarlane GR, Burchett MD (2001) Photosynthetic pigments and peroxidase activity as indicators of heavy metal stress in the grey mangrove, Avicennia marina (Forsk.) Vierh. Mar Poll Bull 42: 233-240

Maksymiec W, Russa R, Urbanik-Sypniewska T, Baszynski T (1994) Effect of excess Cu on the photosynthetic apparatus of runner bean leaves treated at two different growth stages. Physiol Plant 91: 715-721

Peters EC, Gassman NJ, Firman JC, Richmond RH, Power EA (1997) Ecotoxicology of tropical marine ecosystems. Environ Toxicol Chem 16: 12-40

Prasad MNV, Strzalka K (1999) Impact of heavy metals on photosynthesis. In: Prasad MNV, Hagemeyer J (eds) Heavy metal stress in plants: from molecules to ecosystems. Springer – Verlag, Berlin, pp 117-138

Ralph PJ, Burchett MD (1998) Photosynthetic response of *Halophila ovalis* to heavy metal stress. Environ Poll 103: 91-101

SigmaPlot (1997) SigmaPlot, exact graphs for exact science. SigmaPlot 4.0 for Windows, SPSS Inc, USA

Sobrado MA (1999) Drought effects on the photosynthesis of the mangrove, *Avicennia germinans*, under contrasting salinities. Trees 13: 125-130

StatSoft (1995) STATISTICA for Windows, StatSoft Inc, Tulsa, OK

Van Assche FV, Clijsters H (1990) Effects of metals on enzyme activity in plants. Plant Cell Environ 13: 195-206

Vangronsveld J, Clijsters H (1994) Toxic Effects of Metals. In: Farago ME (ed) Plants and the Chemical Elements: Biochemistry, Uptake, Tolerance and Toxicity. VCH Press, Weinheim, pp 149-177