



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu



Acúmulo, distribuição e eliminação de ferro em três espécies vegetais do manguezal

HIULANA PEREIRA ARRIVABENE

Tese apresentada ao Instituto de
Biociências, campus de Botucatu,
UNESP, para obtenção do título de
Doutor no Programa de Pós-Graduação
em Ciências Biológicas (Botânica), AC:
Morfologia e Diversidade Vegetal.

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ARRIVABENE, H.P. **ACÚMULO, DISTRIBUIÇÃO E ELIMINAÇÃO DE FERRO EM TRÊS ESPÉCIES VEGETAIS DO MANGUEZAL**. 2016. 102p. TESE (DOUTORADO) – INSTITUTO DE BIOCÊNCIAS, UNESP - UNIVERSIDADE ESTADUAL PAULISTA, BOTUCATU.

RESUMO – A poluição por metais tem sido um dos principais problemas ambientais, tendo em vista a sua toxicidade, persistência no ambiente e bioacumulação. Os manguezais frequentemente são expostos a esses contaminantes, sendo o ferro um dos metais presentes em maior concentração no sedimento e com papel biogeoquímico importante quanto à precipitação e ciclagem de metais. Investigamos o acúmulo, a distribuição e a eliminação de ferro em *Avicennia schaueriana* Stapf & Leechm. ex Modenke, *Laguncularia racemosa* (L.) C.F. Gaertn. e *Rhizophora mangle* L. submetidas a diferentes concentrações de ferro. Para esse estudo, propágulos das três espécies estudadas foram coletados em campo e cultivados em meio nutritivo de Hoagland. Após o cultivo, as plantas foram submetidas a quatro tratamentos pela adição de 0 (controle), 10, 20 e 100 mg L⁻¹ de Fe(II)SO₄ no meio nutritivo. As plantas foram então coletadas, sendo realizadas análises químicas, anatômicas, histoquímicas e ultracitoquímicas. Parte dessas análises também foi feita em campo para comparação dos dados. Os resultados mostraram que a placa de ferro é local de grande acúmulo de ferro nessas plantas. *L. racemosa* foi a espécie que mais acumulou ferro em placa e raízes, enquanto *A. schaueriana* apresentou os níveis mais altos em caules e folhas. A bioacumulação de ferro em placa e raízes de *L. racemosa* aumentou progressivamente à medida que a concentração de ferro no substrato aumentou. A secreção de ferro por glândulas de sal ocorreu e foi fortemente inibida sob concentrações de ferro acima do controle em *L. racemosa*, enquanto em *A. schaueriana* não houve uma queda evidente entre controle e concentrações de ferro mais elevadas. Em geral, *L. racemosa* apresentou os menores fatores de translocação entre as três espécies estudadas. Com o aumento de ferro no substrato houve redução nos fatores de translocação de ferro entre partes aéreas e raiz e isso foi mais evidente em *R. mangle*. A maioria dos parâmetros analisados na anatomia radicular não mostrou diferença significativa entre os tratamentos. Os parâmetros que mostraram alterações foram espessura de raiz e córtex em *A. schaueriana*, com valores maiores em plantas controle, e espessura de raiz em *L. racemosa*, com valores menores em plantas expostas a 100 mg L⁻¹. Para as três espécies estudadas, os principais locais de bioacumulação de ferro (Fe (II) e Fe (III)) em raízes foram principalmente a epiderme, mas também o córtex. A bioacumulação de ferro em caules e folhas foi diferente para cada espécie. Em caules, o câmbio vascular de *L. racemosa* e a periderme de *R. mangle* foram os principais locais de acúmulo. Por outro lado, em *A. schaueriana* não foi possível identificar os principais locais. Em folhas, o ferro foi detectado principalmente no parênquima aquífero de *A. schaueriana* e no xilema de *L. racemosa*. Ambas as espécies também mostraram acúmulo de ferro em glândulas de sal. Em *R. mangle*, a bioacumulação foliar de ferro ocorreu em diversos tecidos, sobretudo o parenquimático. Análises de material vegetal fresco, recém-coletado em campo, mostraram que glândulas de sal de *A. schaueriana* e *L. racemosa* também exibem reação histoquímica positiva quando testada com o reagente de Perl, indicando acúmulo de ferro. Além disso, ultracitoquimicamente e usando o mesmo marcador específico, o ferro foi localizado em células secretoras,

aderido à membrana de vacúolos e a plasmodesmas. Em ambas as espécies residentes em campo, a presença de ferro na secreção foi confirmada por meio de análise química. Ao nosso conhecimento, este é o primeiro estudo detalhado sobre a resposta de plantas de manguezal a concentrações crescentes de ferro sob condições controladas.

Palavras-chave: glândulas de sal, histoquímica, manguezal, poluição por ferro, ultraestrutura.

ARRIVABENE, H.P. **IRON ACUMULATION, DISTRIBUTION AND ELIMINATION IN THREE PLANT SPECIES OF MANGROVE.** 2016. 102p. PhD. THESIS – BIOSCIENCE INSTITUTE, UNESP – SÃO PAULO STATE UNIVERSITY, BOTUCATU.

ABSTRACT – Metal pollution has long been one of the major environmental problems due to its toxicity, environmental persistence and bioaccumulation. Mangroves are often exposed to these contaminants, and iron is one of the metals present in higher concentrations in sediments and has an important biogeochemical role as regards the precipitation and cycling of metals. We investigate the iron accumulation, distribution and elimination in *Avicennia schaueriana* Stapf & Leechm. ex Modenke, *Laguncularia racemosa* (L.) CF Gaertn. and *Rhizophora mangle* L. exposed to different concentrations of iron. For this study, propagules of all species were collected in field and were cultivated in Hoagland's nutritive medium. After growing, plants were exposed to four treatments by adding 0 (control), 10, 20 and 100 mg L⁻¹ of Fe(II)SO₄ to the nutritive medium. Then, plants were collected and chemical, anatomical, histochemical and ultracitochemical analysis were carried out. Part of these analyses was also made in the field for data comparison. The results showed that the Fe-plaque is a place of high iron accumulation in these plants. *L. racemosa* accumulated the highest amounts of iron in the plaque and roots, while *A. schaueriana* presented the highest levels in stems and leaves. The bioaccumulation of iron in plaque and roots of *L. racemosa* increased progressively as the added-iron concentration rose. The iron secretion through salt glands occurred and it was strongly inhibited in *L. racemosa* at iron concentrations above controls, while *A. schaueriana* did not show a noticeable drop from controls to the highest added-iron concentration used. In general, *L. racemosa* presented the lowest translocation factors among the three studied species. With the increase of iron in the substrate, there was a drop in translocation factors between aerial parts and root, and that was more evident in *R. mangle*. Most analyzed parameters in root anatomy did not show significant changes throughout iron concentrations. The parameters showing changes were the thickness of cortex and root in *A. schaueriana*, with higher values in controls, and thickness of root in *L. racemosa*, with lower values in plants exposed to 100 mg L⁻¹. The main places of iron bioaccumulation (Fe(II) and Fe(III)) in roots were mainly the epidermis, but also cortex, in the three studied species. The bioaccumulation of iron in stems and leaves was different for each species. In stems, the vascular cambium of *L. racemosa* and the periderm of *R. mangle* were the main places for accumulation. Conversely, in *A. schaueriana* it was not possible to verify the main places. In leaves, iron was mostly detected in water storage parenchyma of *A. schaueriana* and in xylem of *L. racemosa*. Both species also showed accumulation of iron in salt glands. The leaf bioaccumulation in *R. mangle* occurred in several tissues, mainly the parenchyma. Fresh plant material analysis, recently sampled in field, shown that salt glands from *A. schaueriana* and *L. racemosa* had positive histochemical reaction when tested with Perl's reagent, indicating iron accumulation. Additionally, ultracitochemistry, using the same specific marker, showed that iron was in secretory cells, adhered to the membrane of vacuoles and to plasmodesmata. In both species resident in field, the presence of iron in the secretion was confirmed by chemical analysis. To our knowledge, this is the first detailed report on the response of mangrove plants to increasing iron concentrations under controlled conditions.

Keywords: histochemistry, iron pollution, mangrove, salt glands, ultrastructure.

Introdução

A poluição por metais contaminantes tem sido um dos principais problemas da atualidade, tendo em vista a sua toxicidade, persistência no ambiente e bioacumulação (Gbaruko & Friday 2007). Esses poluentes representam uma ameaça potencial a vários organismos, afetando negativamente a densidade e diversidade de comunidades bióticas (Hsu *et al.* 2006).

Devido à sua distribuição costeira e proximidade de centros urbanos, os manguezais frequentemente são expostos à poluição por metais (Peters *et al.* 1997, Silva Filho *et al.* 1998, Tam & Yao 1998, Tam & Wong 2000, Defew *et al.* 2005), os quais se acumulam em teores elevados no sedimento. De um modo geral, a alta capacidade de adsorção da matéria orgânica acarreta em baixa biodisponibilidade dos metais acumulados (Lacerda & Abrão 1984, Silva *et al.* 1990). Contudo, em concentrações tão elevadas, uma ínfima fração biodisponível assume uma importância considerável no que diz respeito à bioacumulação de metais em espécies animais e vegetais do manguezal (Szefer *et al.* 1998, Guhathakurta & Kaviraj 2000, MacFarlane *et al.* 2003).

As espécies vegetais do manguezal possuem relativa tolerância aos metais pesados presentes no sedimento (Macfarlane & Burchett 2002) devido a adaptações anatômicas e fisiológicas nas raízes que dificultam a absorção de metais por meio, principalmente, da oxidação do sedimento (Thibodeau & Nickerson 1986, Liu *et al.* 2009), da formação de placas de ferro na superfície radicular (Machado *et al.* 2005) e de barreiras na epiderme e endoderme (MacFarlane & Burchett 2000). Mais recentemente, alguns trabalhos demonstraram que as glândulas de sal presentes nas folhas de algumas espécies de mangue podem secretar metais, tais como zinco (Macfarlane & Burchett 1999, 2000), cobre (Macfarlane & Burchett 2000) e ferro (Arrivabene 2011).

De um modo geral, o ferro é um dos metais em maior concentração no sedimento dos manguezais (Defew *et al.* 2005), sendo que em áreas contaminadas, sobretudo por atividades mineradoras, o nível desse elemento pode aumentar substancialmente. Peters e colaboradores (1997) compilaram, em revisão, valores entre 100 – 33.492 µg/g de Fe no sedimento em áreas diversas de manguezal. No Espírito Santo, estado detentor do maior complexo de produção e exportação de pelotas de ferro do mundo (IBEF 2011), os valores chegam a 23.298 µg/g de Fe (Arrivabene 2015), estando muito acima da maioria dos valores encontrados em outras áreas de manguezal poluídas (Lacerda *et al.* 1989, Silva *et al.* 1990, Sadiq & Zaidi 1994, Sherman

et al. 1998, Sarangi *et al.* 2002, Defew *et al.* 2005). Além disso, o ferro apresenta-se como um dos elementos responsáveis por modular a biodisponibilidade e especiação de metais no sedimento, uma vez que, devido ao ambiente anaeróbico e ao alto teor de matéria orgânica e de sulfeto no sedimento dos manguezais, a formação e precipitação de Fe como sulfetos de ferro contribuem para a alta taxa de sedimentação nesse ambiente (Morse & Rickards 2004).

O ferro é um micronutriente essencial às plantas, envolvido em importantes funções metabólicas, como na biossíntese de clorofila e proteínas (Marschner 2012). Contudo, em níveis muito elevados, esse elemento pode ser tóxico às plantas, podendo acarretar estresse oxidativo e danificar lipídios, proteínas e DNA pela formação de radicais hidroxila na reação de Fenton (Connolly & Guerinot 2002). Quando em excesso, um possível mecanismo para a destoxificação de ferro em plantas tolerantes é sua compartimentalização em vacúolos, onde não reage com substâncias metabolicamente ativas na célula (Kim & Guerinot 2007).

A maior parte dos trabalhos que avaliaram a distribuição do ferro em espécies vegetais do manguezal se concentrou unicamente na análise do teor de ferro foliar (Cuzzuol & Campos 2001, Bernini *et al.* 2006, Agoramoorthy *et al.* 2008). Estudos que avaliem a distribuição do ferro em diferentes órgãos vegetais de espécies de mangue, incluindo raiz, caule e folha, foram desenvolvidos *in situ* (Silva *et al.* 1990, Saifullah *et al.* 2004), sendo encontrado apenas um estudo sobre a bioacumulação de ferro em condições controladas (Cheng *et al.* 2012b). Além disso, estudos anteriores não relataram a distribuição de ferro em todos os compartimentos vegetais. Também não foram encontradas publicações acerca das respostas anatômicas e ultraestruturais de mangues ao ferro contido no sedimento, tampouco um estudo integrado sobre a distribuição desse elemento ao longo dos tecidos vegetais ou, ainda, onde se encontram possíveis barreiras à sua translocação.

Sabendo que as florestas de manguezal são consideradas barreiras biogeoquímicas para o transporte de poluentes em áreas costeiras tropicais (Silva *et al.* 1990) e que essas áreas têm sido consideradas em planos de gestão da poluição como uma alternativa no tratamento de águas residuais urbanas (Silva *et al.* 1990, Tam & Wong 1997), investigações sobre a viabilidade do uso de espécies de mangue na retenção de metais e informações sobre os mecanismos de aprisionamento desses poluentes tornam-se fundamentais.

Além disso, a avaliação do papel funcional de glândulas de sal na eliminação de metais abre uma nova perspectiva de estudo, a qual merece ser investigada em maior profundidade, visto que, tradicionalmente, essas estruturas têm sido descritas unicamente como adaptações à elevada concentração de sal no sedimento (Sobrado 2004, Ye *et al.* 2005).

Diante do exposto, o presente estudo teve como objetivo avaliar, experimentalmente (Fig. 1), o acúmulo, a distribuição e a eliminação de ferro em *Avicennia schaueriana*, *Laguncularia racemosa* e *Rhizophora mangle*, procurando especificamente responder às seguintes questões: (1) Com o aumento do teor de ferro no substrato há um maior acúmulo desse elemento na placa de ferro?; (2) Com o aumento do teor de ferro no substrato há um maior acúmulo desse elemento em raízes, caules e folhas?; (3) Há um maior teor de ferro acumulado em raízes em relação a caules e folhas?; (4) Com o aumento do teor de ferro no substrato há alterações anatômicas nas raízes? (5) Caso sejam encontradas alterações, elas estariam correlacionadas com a formação da placa de ferro e com a quantidade de ferro acumulada nos tecidos vegetais? (6) A epiderme e endoderme radicular constituem os principais locais de acúmulo de ferro na raiz?; (7) Em nível celular, o vacúolo constitui o principal local de acúmulo de ferro?; (8) Há eliminação de ferro pelas glândulas de sal em *A. schaueriana* e *L. racemosa*?; (9) Com o aumento do teor de ferro no substrato há uma maior eliminação desse elemento pelas glândulas de sal?; (8) Há alterações ultraestruturais em glândulas de sal de indivíduos submetidos a maiores concentrações de ferro no substrato?

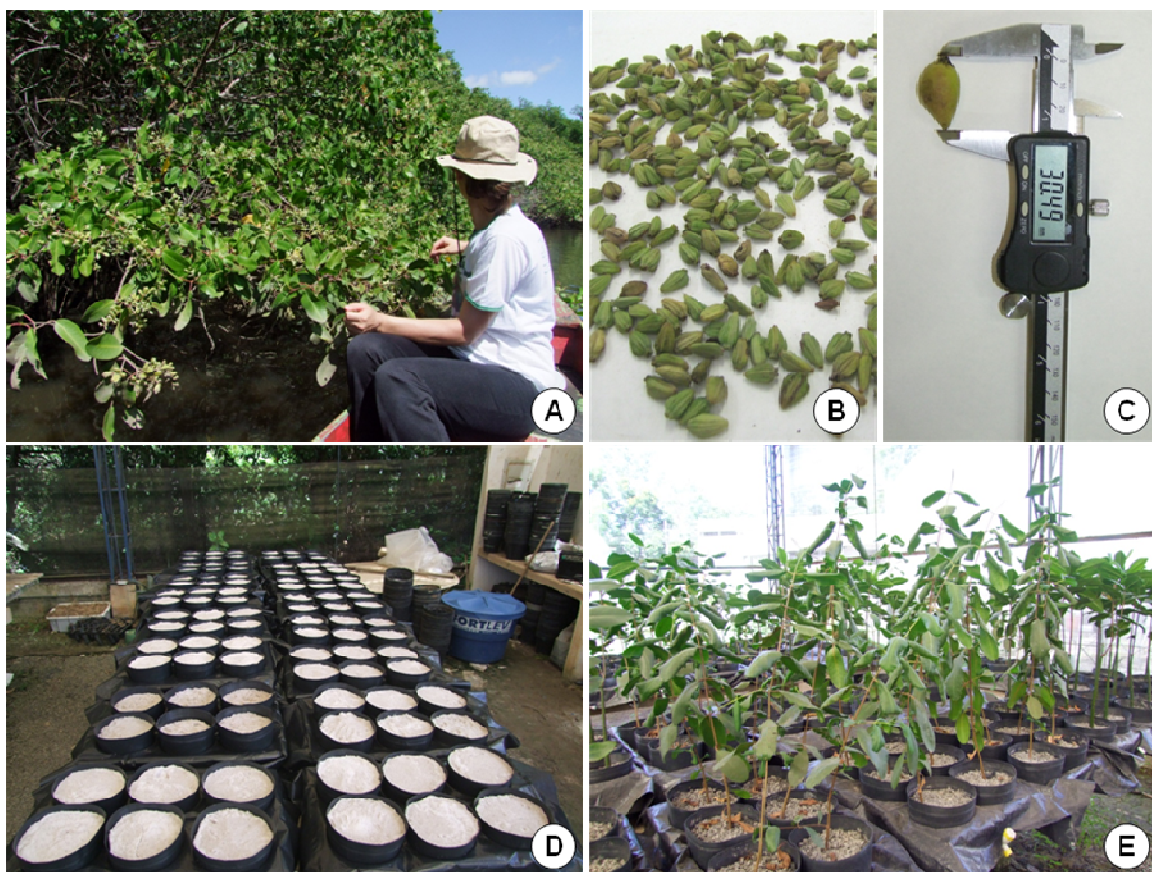


Figura 1. A-E. Etapas do experimento por meio do qual visamos responder às perguntas propostas neste estudo. **A.** Coleta em campo dos propágulos para montagem do experimento. Propágulos de *L. racemosa* (**B**) e *A. schaueriana* (**C**) coletados. **D.** Montagem do experimento em casa de vegetação. **E.** Mudas com 8 meses de idade.

Revisão Bibliográfica

1. O ecossistema manguezal

O manguezal é um ecossistema costeiro que ocorre na interface entre os ecossistemas marinhos e terrestres e está sujeito ao regime de marés. Porque ele ocorre na fronteira entre a terra e o mar, o manguezal é parte do ecossistema contínuo de terrestre a totalmente marinho. Ainda que tenda a reciclar nutrientes e matéria orgânica de forma muito eficiente, ele é um sistema aberto, no sentido de que água, sedimentos, nutrientes e matéria orgânica são importados dele ou exportados para ecossistemas adjacentes pelas entradas e saídas de marés e pelos fluxos de rio, contribuindo com as teias alimentares e transferências de energia (Clough 2013).

Os manguezais encontram-se principalmente ao longo de deltas que recebem grandes quantidades de sedimentos rio acima, embora possam ser encontrados ao longo de costas arenosas e rochosas protegidas, ilhas de coral e até mesmo ao longo de litorais expostos a ondas e correntes marítimas fortes (Clough 2013). Tipicamente, os manguezais apresentam sedimentos argilosos, alagados, com baixo teor de oxigênio, ricos em matéria orgânica e alto conteúdo de sal (Woodroffe 1992).

O termo mangue é usado para descrever o grupo de árvores, arbustos, palmeiras, ervas ou samambaias que crescem nesse ambiente e que exibem um grau acentuado de tolerância a altas concentrações de sal e anoxia do solo, e que produzem propágulos capazes de sobreviver à dispersão pela água do mar (Saenger 2002). As plantas desse ambiente apresentam um conjunto de adaptações, tais como rizóforos que as mantêm nos sedimentos instáveis, pneumatóforos que auxiliam nas trocas gasosas e reciclagem eficiente de nutrientes (Tomlinson 1994). Algumas das adaptações anatômicas e fisiológicas dos mangues relacionadas com a salinidade incluem ultra-filtração dos sais nas raízes (Scholander *et al.* 1962), baixa condutância estomática (Parida *et al.* 2004), aumento da suculência foliar (Suárez & Sobrado 2000, Sobrado 2004, 2005), secreção de sal por meio de glândulas de sal (Sobrado 2004, Ye *et al.* 2005) e acumulação foliar de íons (Hwang & Chen 1995, Suárez & Sobrado 2000, Cram *et al.* 2002). A maior eficiência no uso da água tem sido relatada como o sucesso eco-fisiológico dessas espécies (Sobrado 2005, Ye *et al.* 2005) que, em geral, apresentam maior estabelecimento em baixas concentrações de sal no sedimento (Ball 1998).

Os manguezais são distribuídos predominantemente em regiões tropicais e subtropicais, sendo o maior percentual encontrado entre 5° N e 5° S de latitude (Giri *et al.*

2011). Além da diferente distribuição latitudinal, existe uma diferença na diversidade de espécies, taxonomica e numericamente, entre o Pacífico Indo-Oeste e Leste Africano quando comparado à Costa Atlântica da África Ocidental e das Américas. Existem duas teorias que tentam explicar a diferente biodiversidade dos manguezais no globo: (1) a “hipótese centro de origem” sugere que todos os táxons de mangue desenvolveram-se no Pacífico Indo-Oeste e depois se espalharam para outras partes do mundo e (2) a “hipótese da vicariância” postula que os táxons de mangue originaram em torno do Mar de Tethys e sua diversidade regional foi resultado da diversificação *in situ* após a deriva continental (Ellison *et al.* 1999). Alguns estudos baseados no registro fóssil vegetal e animal têm suportado a segunda teoria, com a aparição dos manguezais ocorrendo no final do Cretáceo (Ellison *et al.* 1999, Sadooni & Al-Saad 2012). A nível local, a composição florística das florestas de mangue é determinada pela variabilidade espacial da elevação e topografia em relação ao nível do mar, pela amplitude das marés, pela proximidade com o mar e pela drenagem local (Clough 2013).

Existem 73 espécies e híbridos de mangue, pertencentes a mais de 20 famílias (Spalding 2010). Segundo Tomlinson (1994) os mangues podem ser divididos em três grupos: elementos principais de manguezal ou mangues verdadeiros, elementos secundários de manguezal e elementos associados de mangue. Os primeiros são caracterizados por ocorrerem exclusivamente no manguezal; desempenharem um papel importante na comunidade e possuírem a capacidade de formar estandes puros; apresentarem especializações morfológicas de adaptação ao ambiente, como raízes aéreas e reprodução por viviparidade e algum mecanismo fisiológico para exclusão de sal; e possuírem isolamento taxonômico de parentes terrestres ao nível de família ou subfamília. Os elementos secundários de manguezal não possuem a habilidade de formar elementos conspícuos da vegetação, enquanto os associados de mangue nunca habitam comunidades de mangues verdadeiros, ocorrendo somente na vegetação transicional com outros ecossistemas.

Considerando esta classificação, os mangues verdadeiros brasileiros possuem como representantes da flora arbórea típica três gêneros (*Avicennia* L., *Laguncularia* Gaertn. e *Rhizophora* L.) e seis espécies (*Rhizophora mangle* L., *Rhizophora racemosa* G.Mey, *Rhizophora harrisonii* Leechm., *Avicennia germinans* (L.) L., *Avicennia schaueriana* Stapf & Leechm. ex Moldenke e *Laguncularia racemosa* (L.) C.F.Gaertn.) (Kjerfve & Lacerda 1993). No Brasil, as áreas de manguezal cobrem uma área aproximada de 962.683 ha, sendo o terceiro país mais rico em manguezal no mundo, com 7% do total global das florestas (Giri *et al.* 2011).

Os manguezais fornecem uma série de bens e serviços, incluindo matérias-primas e alimentos, manutenção da pesca, turismo, recreação, educação e pesquisa. No contexto da proteção do ambiente, os manguezais estabilizam o sedimento fino evitando a erosão costeira, além de atuar como uma barreira natural, reduzindo os efeitos de tempestades e inundações. Os manguezais também desempenham um papel crucial na qualidade da água, pela assimilação e reciclagem de resíduos, protegendo recifes de corais, leitos de algas marinhas e águas importantes de navegação contra o assoreamento e a poluição, além do seu papel na estabilidade do clima, pelo sequestro de carbono, uma vez que estão entre os ecossistemas mais produtivos e biogeoquimicamente ativos. Além disso, esse ecossistema único tem a função de promover a biodiversidade, suportando uma ampla variedade de vida selvagem (Barbier *et al.* 2011, Vo *et al.* 2012). Para comunidades costeiras que convivem com o manguezal, o uso tradicional dos recursos é conectado intimamente com a saúde e o funcionamento do sistema, sendo frequentemente regido por tradições e heranças ligadas à cultura tradicional (Walters *et al.* 2008). O valor econômico dos manguezais foram estimados entre US\$ 10.000 ha⁻¹ e US\$ 35.921 ha⁻¹, diferenças estas devidas à especificidade geográfica e temporal, onde o mesmo ecossistema pode ter valores diferentes em diferentes áreas geográficas causadas por distintas atividades econômicas e culturais (Vo *et al.* 2012). Em todo o mundo, os serviços ambientais prestados pelas florestas de mangue são estimados em pelo menos US\$ 1,65 bilhões por ano (Costanza *et al.* 1997).

Apesar de todos os bens e serviços fornecidos à humanidade, os manguezais apresentam as taxas de perda de cobertura mais rápida dos ecossistemas em todo o mundo, sendo estimada em 35% a área de florestas de mangue perdida somente entre os anos 1980 e 2000 (Valiela *et al.* 2001). Dentre os impactos sofridos pelos manguezais estão a poluição por metais contaminantes, petróleo e óleos dispersantes, bem como a exploração de madeira e a conversão de áreas para aquicultura, agricultura e salinas. A expansão urbana costeira, portuária, industrial e turística, juntamente com o descaso dos gestores, tem sido responsável por perdas significativas de áreas previamente ocupadas por esse ecossistema (Clough 2013).

1.1. Poluição por metais

Identificar a origem dos metais presentes no sedimento dos manguezais muitas vezes é difícil, uma vez que sua presença é uma combinação de fontes naturais e antropogênicas. Metais de origem antropogênica podem entrar no sistema por meio do transporte de rios, intrusão de água marinha ou deposição atmosférica (Bayen 2012). Uma vez introduzidos, os

manguezais podem reter eficientemente os metais no sedimento e prevenir sua distribuição para ecossistemas adjacentes, sendo por isso considerados barreiras biogeoquímicas ao transporte desses contaminantes em áreas costeiras (Zhou *et al.* 2010). Além disso, o alto teor de matéria orgânica e partículas finas, característicos do sedimento do manguezal, contribuem para a retenção de metais no ambiente (Machado *et al.* 2008, Zhou *et al.* 2011). De fato, altos níveis de metais contaminantes têm sido relatados no sedimento dos manguezais em várias áreas do globo (Lewis *et al.* 2011, Bayen 2012). Águas residuais industriais geralmente contém teores elevados de metais (Barakat 2011) e o escoamento de herbicidas em áreas agrícolas a montante também contribui para o agravamento deste problema (Gimeno-Garcia *et al.* 1996).

Os níveis de metais podem variar dentro do manguezal de acordo com a profundidade, distância da costa, tipo de vegetação e hidrologia, enquanto a biodisponibilidade e especiação dos metais acumulados no sedimento depende de uma gama de fatores ainda maior. Dentre esses podemos citar associação com a matéria orgânica, sorção sobre partículas finas, condições redox e efeitos da salinidade, bem como componentes bióticos (como tipo de vegetação e micro e macrofauna associada), os quais têm influência sobre esses processos físico-químicos (Bayen 2012). O entendimento da biodisponibilidade desses contaminantes é complexo, uma vez que é afetado por pelo menos 22 fatores geoquímicos, hidrológicos e físico-químicos (Lewis *et al.* 2011). Assim, o destino do metal nos sedimentos é governado por inúmeros processos, incluindo sorção/dessorção, precipitação/dissolução e complexação/descomplexação, de modo que os elementos apresentam-se nas seguintes formas químicas: (1) metais solúveis em água, como íons livres ou inorgânicos ou orgânicos complexos; (2) metais trocáveis; (3) metais precipitados como compostos inorgânicos, incluindo sulfetos insolúveis; (4) metais complexados com materiais húmicos de grande peso molecular; (5) metais adsorvidos ou ocluídos à oxi-hidróxidos precipitados; (6) metais ligados dentro da estrutura de rede cristalina de minerais primários (Du Laing *et al.* 2009).

1.2. Adaptações anatômicas e fisiológicas das plantas aos metais

A acumulação de metais nas plantas deve ser considerada como um resultado combinado entre a especiação e biodisponibilidade dos metais no sedimento e as adaptações anatômicas e fisiológicas das plantas associadas à prevenção de sua bioacumulação.

Plantas de manguezal possuem relativa tolerância aos metais presentes no sedimento (Macfarlane & Burchett 2002), sendo registrados baixos fatores de bioconcentração para

grande parte dos metais (Machado *et al.* 2002, Sarangi *et al.* 2002, Saifullah *et al.* 2004). A capacidade de manter a carga de metal a um baixo potencial de remobilização e captação biótica (Machado *et al.* 2002) é conseguida, principalmente, pela perda radial de oxigênio (ROL, *radial oxygen loss*) a partir das raízes. Como a sobrevivência das plantas em um sedimento anaeróbico depende do fornecimento adequado de oxigênio através do aerênquima radicular, o oxigênio armazenado e transportado dentro desse tecido ou é consumido pelas células, ou é difundido em direção ao ápice radicular ou é perdido para a rizosfera, sendo este último denominado ROL (Armstrong 1979). Quando ROL ocorre, permite às raízes criarem uma rizosfera aeróbica, onde produtos tóxicos do metabolismo microbiano anaeróbico podem ser oxidados, diminuindo, assim, sua biodisponibilidade (Tripathi *et al.* 2014). Muitas espécies conseguem evitar a perda excessiva de oxigênio para a rizosfera formando uma 'barreira' completa ou parcial nas camadas da epiderme, exoderme ou subepiderme, sendo que o tipo de barreira, a extensão de ROL e a área de aerênquima no córtex variam ao longo da raiz e essas variações são específicas para cada espécie (Pi *et al.* 2009). Além disso, as plantas de manguezal podem alterar o padrão espacial da barreira à ROL, de modo a absorver menos metal e, assim, serem mais tolerantes aos compostos potencialmente tóxicos (Cheng *et al.* 2010, 2012a).

Com ROL, parte dos metais oxidados na rizosfera são, ainda, precipitados junto à superfície radicular, sendo essa capa de metais conhecida como placa de ferro, uma vez que possui, dentre outros metais, compostos ricos em oxi-hidróxidos de ferro (Tripathi *et al.* 2014). A placa de ferro pode servir como barreira física à entrada de contaminantes na planta e tem sido reconhecida como um importante mecanismo capaz de evitar a entrada excessiva de metais em plantas de ambientes alagados (Cheng *et al.* 2014b, Tripathi *et al.* 2014).

Na raiz, a epiderme fornece a principal barreira ao transporte de metais, mas a endoderme também apresenta importante contribuição, como observado para Cu, Zn, Pb e Cd em *Kandelia obovata* (Lu *et al.* 2014) e Cu, Pb e Zn em *Avicennia marina* (MacFarlane & Burchett 2000). Foi observado *in situ* que a periderme radicular de mangues é mais espessa em locais mais contaminados por metais (Souza *et al.* 2014a, 2014b, 2015), havendo inclusive uma correlação negativa entre a espessura da periderme com o nível de As acumulado em raízes de *L. racemosa* (Souza *et al.* 2014a) e com o nível de Zn acumulado em raízes de *A. schaueriana* (Souza *et al.* 2015). Ainda em raízes, foi documentado que os índices de tolerância dos mangues aos metais são positivamente correlacionados com as quantidades de

deposição de lignina e suberina em paredes celulares da exoderme, uma vez que a captação de metais é retardada pela presença desses compostos (Cheng *et al.* 2014a).

Uma vez absorvidos, os metais tendem a se acumular em quantidades muito maiores nas raízes quando comparados à parte aérea (Lu *et al.* 2014, Naidoo *et al.* 2014). Nas folhas, foi comprovado que as glândulas de sal, presentes em algumas espécies, podem eliminar Cu, Zn (Macfarlane & Burchett 2000, Naidoo *et al.* 2014) e Fe (Arrivabene 2011), talvez contribuindo para o menor acúmulo foliar desses metais.

Estudos subcelulares mostraram que a compartimentalização dos metais em espécies de mangue ocorre principalmente na parede celular (MacFarlane & Burchett 2000, Weng *et al.* 2012, Lu *et al.* 2014) e no vacúolo (Hossain *et al.* 2012), sendo esses considerados os principais locais de detoxificação desses contaminantes.

2. Ferro: função, transporte e regulação em plantas

Entre os micronutrientes essenciais para as plantas, o ferro (Fe) é necessário em maior abundância. Ele funciona em vários processos importantes, incluindo a fotossíntese, a respiração, a biossíntese de clorofila, além de ser componente em proteínas heme, Fe-enxofre e outras enzimas (Marschner 2012).

O ferro é o segundo elemento mais abundante da crosta terrestre, porém sua solubilidade é extremamente baixa, principalmente em solos alcalinos. Em sistemas aerados, as concentrações dos íons Fe são menores que 10^{-5} M devido à formação de óxidos, hidróxidos e oxihidróxidos de Fe (Marschner 2012).

Quelatos de Fe(II) e Fe(III) são as formas dominantes de Fe solúvel no solo. Como um elemento de transição, Fe é caracterizado pela mudança em seu estado de oxidação $\text{Fe}^{2+} \leftrightarrow \text{Fe}^{3+}$ e pela sua habilidade em formar complexos octaédricos com vários ligantes. Dependendo do ligante, o potencial redox de Fe (II/III) varia amplamente. Esta variabilidade explica a importância dos estados redox de Fe em sistemas biológicos. Contudo, em sistemas aeróbicos, quando o Fe existe em um estado livre ou com muitos quelatos de baixo peso molecular, essa mesma propriedade o torna propenso a geração de espécies reativas de oxigênio (ROS, *reactive oxygen species*), tais como radical superóxido ou radical hidroxila e compostos relacionados, por exemplo (Marschner 2012):



ou na reação de Fenton:



ou não reação de Haber-Weiss:



Esses radicais são altamente tóxicos e responsáveis pela oxidação de ácidos graxos poli-insaturados de lipídios e proteínas de membrana. A prevenção do dano oxidativo tem sido alcançada pela forte ligação ou incorporação do Fe a estruturas (por ex. proteínas heme e não-heme), as quais permitem serem controladas por reações reversíveis de oxi-redução $\text{Fe(II)} \leftrightarrow \text{Fe(III)}$, incluindo aquelas de proteção oxidante (Marschner 2012).

A ativação da absorção de ferro é um perigo duplo: não só o ferro em si é tóxico, mas os sistemas de captação de ferro são mal seletivos e permitem a entrada de outros metais potencialmente tóxicos. As plantas, por conseguinte, desenvolveram mecanismos para lidar com a entrada concomitante de metais tóxicos. Recentemente, um importante progresso na compreensão dos genes envolvidos na absorção de ferro pelas plantas, neutralização de metais tóxicos que entram juntamente com esse metal e distribuição de ferro para organelas tem sido feito (Thomine & Vert 2013).

O controle rígido da captação, da utilização e do armazenamento de Fe em resposta à disponibilidade ambiental permite às plantas adquirir Fe suficiente, evitando sua toxicidade. Os progressos recentes na regulação do Fe têm se concentrado em *Arabidopsis thaliana* e arroz, que geralmente representam sistemas não-gramíneas e gramíneas, respectivamente. As plantas desenvolveram duas estratégias para adquirir ferro da rizosfera: a de redução de plantas não-gramíneas (estratégia I) e a de quelação de gramíneas (estratégia II) (Kobayashi & Nishizawa 2012, Thomine & Vert 2013).

Os dois processos principais na Estratégia I são a redução de quelatos férricos na superfície da raiz e a absorção dos íons ferrosos gerados através da membrana plasmática da raiz. Outros processos envolvidos na Estratégia I incluem excreção de prótons e compostos fenólicos a partir das raízes para a rizosfera, que acredita-se aumentar a solubilidade de íons férricos ou ajudar na capacidade de redução de Fe férrico na superfície da raiz. A indução destes componentes moleculares sob baixa disponibilidade de Fe é acompanhada por alterações morfológicas na arquitetura da raiz, tais como a formação de células de transferência e pelos radiculares extras. A resposta envolvida na Estratégia II baseia-se na biossíntese e secreção de fitosideróforos da família dos ácidos muginéicos (MA, *mugineic acids*), que são específicos para plantas gramíneas. Os MAs secretados na rizosfera, solubilizam Fe (III), e os complexos Fe (III)-MA resultantes são retomados nas células radiculares pelos transportadores (Kobayashi & Nishizawa 2012). Componentes-chave,

incluindo transportadores moleculares, enzimas e agentes quelantes foram esclarecidos para ambas as estratégias, e muitos desses componentes devem funcionar também no interior da planta facilitando o transporte interno de ferro. O transporte intracelular de ferro também está sendo esclarecido. A maioria dos genes que codificam estes componentes são transcricionalmente regulados em resposta a disponibilidade de ferro e a regulação do gene é um passo crucial para o enfrentamento em ambientes flutuantes (Kobayashi & Nishizawa 2012, Thomine & Vert 2013).

Devido à fraca solubilidade e elevada reatividade do Fe, a sua translocação dentro do corpo da planta deve ser associada a moléculas quelantes e ao controle adequado dos estados redox entre as formas ferrosa e férrica. A translocação de Fe envolve várias etapas, incluindo o transporte radial entre os tecidos da raiz; o transporte simplástico pelas estrias de Caspary; o transporte, carregamento e descarregamento do xilema; a transferência xilema a floema; o transporte, carregamento e descarregamento do floema; o movimento simplástico em direção ao local da procura; e a retranslocação da fonte ou do tecido em senescência (Kim & Guerinot 2007). Estudos indicam que, entre os principais agentes quelantes dentro do corpo da planta, estão o citrato, a nicotianamina e MAs (Kobayashi & Nishizawa 2012).

Uma vez dentro da célula, o Fe deve ser entregue a compartimentos apropriados para sua utilização na função celular e para evitar que se acumule em excesso, o que poderia levar à toxicidade. O cloroplasto é o local de maior acúmulo de Fe em células vegetais, acumulando cerca de 80% - 90% do Fe celular, onde há grande demanda por Fe no aparato fotossintético. Plastídios e mitocôndrias são não só locais para reações enzimáticas e de transporte de elétrons mediados por Fe, mas também local de biogênese para o aglomerado Fe-enxofre. Além disso, o vacúolo geralmente se apresenta como um local de grande acúmulo de metais, evitando sua toxicidade (Marschner 2012).

3. Descrição das espécies em estudo

***Avicennia schaueriana* Stapf & Leechm. ex Moldenke (Acanthaceae)**

Conhecida popularmente como mangue preto ou siriúba, pertence à família Acanthaceae, a qual possui distribuição predominantemente pantropical e inclui cerca de 200 gêneros e 3000 espécies (Souza & Lorenzi 2008). No Brasil ocorrem 40 gêneros e 449 espécies (Profice *et al.* 2015).

Descrição da espécie: Raízes subterrâneas extensas, suportando pneumatóforos e raízes de absorção. Casca variavelmente áspera, escura, rígida e fissurada. Lâmina foliar

inteira, coriácea ou um pouco carnosa com nervuras inconspícuas e nervura central proeminente na face abaxial. Lâminas foliares variando de ovadas, elípticas a estreitamente lanceoladas; ápice arredondado, ligeiramente emarginado; base aguda. Presença de glândulas de sal em ambas as faces da lâmina foliar (Tomlinson 1994).

***Laguncularia racemosa* (L.) C.F. Gaertn. (Combretaceae)**

Conhecida popularmente como mangue branco, pertence à família Combretaceae, a qual possui distribuição predominantemente pantropical e inclui cerca de 20 gêneros e entre 400 e 500 espécies (Souza & Lorenzi 2008). No Brasil ocorrem 5 gêneros e 62 espécies (Marquete *et al.* 2015). *Laguncularia* é um gênero monotípico de mangue com distribuição restrita nas Américas e no Oeste da África (Tomlinson 1994).

Descrição da espécie: Raízes subterrâneas extensas, como cabo, suportando pneumatóforos e raízes de absorção. Casca áspera, fissurada, cinza. Ramos com numerosas lenticelas, ligeiramente proeminentes. Lâmina foliar um pouco carnosa, elíptica a oblonga, margem inteira, arredondada a ligeiramente emarginada apicalmente. Base truncada e glabra. Folhas com pecíolo curto, apoiando duas glândulas circulares na superfície adaxial da lâmina (Tomlinson 1994). Francisco e colaboradores (2009) reconheceram 5 tipos de estruturas glandulares: um par de nectários extraflorais na porção adaxial do pecíolo; glândulas “tipo vulcão”, secretoras de sal, distribuídas por toda a superfície abaxial e adaxial da lâmina foliar; micro-pêlos na face abaxial, distribuídos desde a metade distal até o ápice da lâmina foliar, e sobre a nervura mediana; hidatódios na periferia da folha sobre as nervuras secundárias no lado abaxial da lâmina foliar; e glândulas do bordo, imbricadas ao redor de toda a margem foliar.

***Rhizophora mangle* L. (Rhizophoraceae)**

Conhecida popularmente como mangue vermelho, pertence à família Rhizophoraceae, a qual possui distribuição pantropical e inclui 16 gêneros e cerca de 150 espécies (Souza & Lorenzi 2008). No Brasil ocorrem 4 gêneros e 10 espécies (Mansano *et al.* 2015). A tribo Rhizophoreae inclui 4 gêneros exclusivos de mangue (*Bruguiera*, *Ceriops*, *Kandelia* e *Rhizophora*), nos quais a condição vivípara é uma particularidade (Tomlinson 1994).

Descrição da espécie: Presença de rizóforos com geotropismo positivo, responsável por fornecer estabilidade no sedimento inconsistente, os quais, quando em contato com o solo, formam as raízes (Menezes 2006). Folhas simples, inteiras, elípticas, com margens um pouco

recurvadas. Textura foliar coriácea, glabra, mas com numerosos pontos suberizados na superfície abaxial, visíveis em folhas mais velhas como pontos escuros. Nervuras evidentes, mas não proeminentes. Pecíolo curto e cilíndrico. Sementes vivíparas, germinadas pela extensão do hipocótilo; propágulos com 15-20 cm, sem lenticelas conspícuas (Tomlinson 1994).

Conforme estabelecido pelo Programa de Pós-Graduação em Ciências Biológicas (Botânica) do IBB, UNESP, os resultados obtidos durante a execução deste projeto de Doutorado foram reunidos em artigos científicos para publicação.

Artigo 1 – “Differential bioaccumulation and translocation patterns in three mangrove plants experimentally exposed to iron. Consequences for environmental sensing” submetido ao periódico Environmental Pollution (A1, avaliação Qualis Capes).

Artigo 2 – “Iron elimination through salt glands in leaves of two mangrove species” a ser submetido ao periódico The Science of Nature - Naturwissenschaften (A1, avaliação Qualis Capes).

Artigo 1

Differential bioaccumulation and translocation patterns in three mangrove plants experimentally exposed to iron. Consequences for environmental sensing.

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Abstract

Avicennia schaueriana, *Laguncularia racemosa* and *Rhizophora mangle* were experimentally exposed to increasing levels of iron (0, 10, 20 and 100 mg L⁻¹ added Fe(II) in Hoagland's nutritive medium). The uptake and translocation of iron from roots to stems and leaves, Fe-secretion through salt glands (*A. schaueriana* and *L. racemosa*) as well as anatomical and histochemical changes in plant tissues were evaluated. The main goal of this work was to assess the diverse capacity of these plants to detect mangroves at risk in an area affected by iron pollution (Vitoria, Espírito Santo, Brazil). Results show that plants have differential patterns with respect to bioaccumulation, translocation and secretion of iron through salt glands. *L. racemosa* showed the best environmental sensing capacity since the bioaccumulation of iron in both Fe-plaque and roots was higher and increased as the amount of added-iron rose. Fewer changes in translocation factors throughout increasing added-iron were observed in that species. Furthermore, the amount of iron secreted through salt glands of *L. racemosa* was strongly inhibited when exposed to added-iron. Between the three species, *A. schaueriana* showed the highest levels of iron in stems and leaves. On the other hand, *R. mangle* presented low values of iron in these compartments. Even so, there was a significant drop in the translocation factor between aerial parts with respect to roots, since the bioaccumulation in plaque and roots of *R. mangle* increased as iron concentration rose. Moreover, rhizophores of *R. mangle* did not show changes in bioaccumulation throughout the studied concentrations. So far, we propose *L. racemosa* as the best species for monitoring iron pollution in affected mangroves areas. To our knowledge, this is the first detailed report on the response of these plants to increasing iron concentration under controlled conditions, complementing existing data on the behavior of the same plants under field exposure.

Keywords:

Histochemistry, iron bioaccumulation, iron plaque, iron translocation, mangrove pollution, pollution sensing.

Highlights:

- First detailed evaluation on the response of mangrove plants exposed to iron pollution.
- *Laguncularia racemosa* presented the best capacity for sensing iron pollution.
- *Rhizophora mangle* had a drop in iron translocation factors throughout increasing iron.
- *Avicennia schaueriana* showed the highest levels of iron in stems and leaves.

1. Introduction

Mangroves are part of the ecosystem continuum from terrestrial to fully marine environments that occur at the boundary between land and sea, and are flooded regularly by the tide (Clough, 2013). Coastal wetlands have been recognized for their ability to stabilize shorelines and to protect coastal communities. Coastal wetland vegetation is an effective shoreline buffer, which acts as a natural barrier to stabilize fine sediment, preventing coastal erosion. Moreover, they reduce effects of storms and flooding, maintain water quality and biodiversity, and support a wide range of wildlife. Mangroves may have an indirect value in the protection of coastal property and economic activities such as fishery (Barbier et al., 2011; Vo et al., 2012). However, despite all the goods and services provided around the world, estimated at \$1.7 to \$2.8 billion per year (Brander et al, 2012; Costanza et al., 1997), mangroves have the fastest rates of loss of ecosystems worldwide, and are increasingly impacted by pollution (Valiela et al., 2001). Among the contaminants, pollution by metals is a concern, since these elements can be uptaken, transferred and involved in metal biomagnification along the food chain, generating toxicity for the biota (Cardwell et al, 2013; Nica et al, 2012).

Metal accumulation in mangrove sediment is favored by its high capacity of absorption of organic matter (OM) and small particles (Zhou et al., 2010). The anaerobic environment, in addition to high levels of OM and iron sulfide, in mangrove sediments enhance iron settling, and the accumulation of metals. Therefore, iron is responsible for modulating the bioavailability and redox characteristics of metals in sediments (Morse and Rickard, 2004).

Vascular plants mangroves are crucial to the dynamics of the estuarine ecosystem, strongly influencing the processes of metals retention, with unique biological mechanisms. Some mechanisms involved in the resistance of mangrove plants to metals, namely the

accumulation of metals in roots in comparison with the aerial parts of the plants, have been reported (Nath et al., 2014). These mechanisms involve the formation of an iron (Fe) plaque on the root surface (Cheng et al., 2014; Du et al., 2013), and metal retention in the root (epidermis and endodermis) (Lu et al. 2014; MacFarlane and Burchett, 2000). Evidence suggests that the extent of Fe-plaque formed on mangrove root is species-specific (Pi et al. 2011) and that the amount of Fe-plaque can be related to adaptive changes in the root anatomy in response to pollution (Pi et al., 2010). An additional adaptive response in some mangrove plants is the secretion of metals through salt glands (Macfarlane and Burchett 1999; 2000; Naidoo et al. 2014).

Avicennia schaueriana Stapf & Leechm. ex Moldenke, *Laguncularia racemosa* (L.) C.F. Gaertn. and *Rhizophora mangle* L. are mangrove species, commonly found in Brazil (Giri et al., 2011; Tomlinson, 1994). These plants are well adapted to saline wetlands, presenting aerenchymatose roots with air gaps that are usually bigger in *A. schaueriana* and *L. racemosa* in comparison with *R. mangle*, which additionally shows warming root cells (Menezes, 2006; Youssef and Saenger, 1996). These three species show different ways to cope with the saline sediment. *A. schaueriana* and *L. racemosa* are species with salt glands in leaves; conversely, *R. mangle* have glabrous leaves (Tomlinson, 1994). Mangrove plants secreting salt through salt glands are capable of absorbing more salt through the root (salt-including species), which has been proposed as a mechanism that facilitates the absorption of metals, resulting in a higher bioaccumulation in these plants (Bernini et al., 2006; Cuzzuol and Campos, 2001; Sarangi et al., 2002).

The presence of iron mines close to mangrove areas can result in an additional load of metals in the sediment; particularly iron (Veerasingam et al., 2015). The state of Espírito Santo (Brazil) has the largest iron production of the country, being the biggest mining harbor in Brazil (IBEF, 2011). Levels of iron in sediments of this area reach 23 mg g⁻¹ (Arrivabene et

al., 2015), which are above levels found in less polluted mangrove areas (Defew et al., 2005; Lacerda et al., 1989; Sadiq and Zaidi, 1994; Sarangi et al., 2002; Sherman et al., 1998; Silva et al., 1990).

Some field studies have been carried out to evaluate the bioaccumulation of iron in mangrove plants (Souza et al. 2014a, 2014b; 2015). However, to our knowledge, there are few studies on the bioaccumulation of iron under controlled conditions (Cheng et al. 2012), looking to isolate the behavior of this metal from the complex matrix present in mangrove sediments, like the presence of different amounts of organic matter, dissolved oxygen, granulometry, etc. (Arrivabene et al., 2015). Furthermore, previous studies did not report the iron distribution in all plant compartments. It is worth to mention that, in addition to being a micronutrient, iron can reach concentrations toxic to the plant if its amount in sediments as well as its bioavailability are high enough (Kobayashi et al., 2012).

Considering the above-described evidence, the main goal of this work was to evaluate the diverse capacity of these three plants to absorb, translocate and bioaccumulate iron in different plant compartments, as well as to evaluate changes in the plant anatomy. Thus, we look to verify if these plants can be used as pollution sentinels in an area with mangroves at risk because of iron pollution (Vitoria, Espírito Santo, Brazil).

2. Materials and Methods

2.1. Experimental set up

Propagules of *A. schaueriana*, *L. racemosa* and *R. mangle* were collected at the ecological reserve of the Lameirão county (Estação Ecológica Municipal Ilha do Lameirão), State of Espírito Santo, Brazil, during the spring 2013 (*A. schaueriana*), or the summer 2014 (*L. racemosa* and *R. mangle*). Propagules were transported to a greenhouse at the Federal University of Espírito Santo, where they were cultivated in pre-cleaned PVC pots (2.8 L each)

containing washed sand. Sand pots were stored in receptacles containing a nutritive medium (Hoagland and Arnon, 1950), with 0.25 ionic strength and a salt content of 7 g L⁻¹. The level of the nutritive medium was *ca.* 3 cm during plant grow, and *ca.* 7 cm during exposures, simulating mangrove conditions without the presence of organic matter. The nutritive medium was covered with a black PVC film to prevent photo-oxidation.

Propagules were developed during eight months, afterward plants were used for exposure. Exposures were performed by adding 0 (control), 10, 20 and 100 mg L⁻¹ Fe(II)SO₄ (to simulate the bioavailable form of iron), disodium EDTA and MES buffer (1mM, pH 6) to the nutritive medium. Iron concentrations of 10 and 20 mg L⁻¹ were selected as they are close to values found in the interstitial water during field studies in the mangrove area of Espírito Santo (Arrivabene et al., 2015). The highest concentration (100 mg L⁻¹) was selected to simulate a more toxic condition, with iron levels exceeding current environmental levels, similar to the levels found during an incident like the recent mine dam break in the neighbor State of Minas Gerais (Brazil). The nutritive medium was renewed weekly.

Sets of five independent plants (n=5) from each species (randomly selected) were exposed to different iron concentrations during eight weeks. After exposure, plants were harvested and analyzed as described below.

2.2. Chemical analysis of iron

Glassware and plasticware used for sampling preparation and analysis were previously washed using a neutral detergent Extran MA 02 (5% v/v), 15-50% v/v nitric acid (63.7%) sub-boiling grade and ultrapure water (<5 µg L⁻¹ TOC). Ultra-pure water was obtained from a purification system Arium 61316-RO, plus Arium 611 UV (Sartorius, Germany). Nitric acid sub-boiling grade was prepared from analytical grade acid, using a sub-boiling distiller

(Figmay, Córdoba, Argentina). The purity of the nitric acid was checked by Mass Spectrometry Inductively Coupled Plasma (ICP-MS) (Agilent 7500cx, USA).

Iron concentrations were determined in triplicate by ICP-MS, using an ASX-100 autosampler (CETAC Technologies, Omaha, NE), according to USEPA (2009) methods. Quality assurance (QA) and quality control (QC) were performed using a certified reference material (CRM): peach leaves NIST SRM 1547. Recoveries from CRMs were above 80% as recommended by USEPA. Spiked samples were also prepared for roots, stems, rhizophores and leaves to verify the matrix effect. Thus, standard Merck VI CertiPUR® (Merck Química, Buenos Aires, Argentina) was added to dried plant materials; the rest of the procedure was the same as used for non-spiked samples. The average recovery of these assays ranged from 92 to 105%.

2.2.1. Iron in plaque

The extraction of the Fe-plaque was performed using the dithionite-citrate-bicarbonate technique (DCB), which is effective in extracting the iron retained in the Fe-plaque present along the root surface of plants. There are different reports on the proportion of reagents used in this method (Liu et al., 2004; Otte et al., 1991; Taylor and Crowder, 1983). Thus, we carried out a comparison between these three methods (Table 1) to verify the best experimental set up to be used with our samples. From Table 1, we can see that the method proposed by Taylor and Crowder (1983) allows the highest extraction of iron from the Fe-plaque, while no significant differences were found for total iron (Fe-plaque + internal root) among the three methods. Therefore, we used the protocol reported by Taylor and Crowder (1983) to extract the Fe-plaque. Briefly, the entire root system was washed with reverse osmosis water (RO-water) and air-dried. Next, an amount equivalent to 0.2 g dry root was stirred at room temperature for 3 h in a cold DCB solution (40 mL of 0.3M $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$;

5 mL of 1M NaHCO₃, and 3 g de Na₂S₂O₄) (Taylor and Crowder, 1983). The approximate ratio between fresh and dry mass of roots was analyzed previously in roots from different individuals. After DCB extraction, roots were rinsed with RO-water, dried in an oven (37°C) until constant weight. The DCB extraction solution was filtered using nitrocellulose 0.45 µm filters (Millipore, USA), acidified with ultra-pure HNO₃, and stored at 4°C until analysis.

2.2.2. Iron in roots, stems, rhizophores and leaves

Roots, stems, rhizophores and leaves samples were washed with RO-water, dried at 37°C until constant weight, grinded and homogenized with a mortar. All samples were digested according to Chappaz et al. (2012) with slight modifications, using 4 mL nitric acid (70% ultra-pure, sub-boiling grade) and 1 mL HCl (37%, ultra-pure sub-boiling grade), in pre-cleaned PTFE tubes (Sevillanex, USA) at constant temperature during 24 h. All samples were filtered (0.45 µm, Millipore, USA) and stored at 4°C until analysis. Controls were prepared using the same protocol without sample (only reagents).

Translocation factors (Tf) were calculated between different plant compartments: TFsr: [Fe]-stem/[Fe]-root; TFIs: [Fe]-leaves/[Fe]-stem; TFIsr: [Fe]-leaves/[Fe]-root; and TFsh: [Fe]-(leaf + stem)/[Fe]-root.

2.2.3. Iron in the secretion from salt glands (*A. schaueriana* and *L. racemosa*).

The entire procedure was performed within a PVC cubic structure, protecting leaves from possible atmospheric contamination by particulate iron, including dust. Leaves from the third node were successively washed with 5% v/v Extran MA 02 neutral detergent and then with RO-water. The PVC cubic structure was wrapped using a transparent PCV film, containing small orifices to prevent saturation with water vapor, and minimize interference with the photosynthetically active radiation on the leaves, which could cause the stomata

closure and consequent reduction in the flow of transpiration and secretion (as observed by Scholander et al., 1962). After three days, leaves were collected and immersed in DCB solution for 3 h (Taylor and Crowder, 1983). Leaves were also used to determine glandular density ($\text{n}^\circ \text{mm}^{-2}$) by the printing technique, using a drop of cyanoacrylate ester adhesive (Super-Bonder®) in a histological slide. Thus, the obtained foliar surface was analyzed by microscopy (Nikon E200 microscope, Tokyo, Japan). Six random optical fields were measured from each foliar surface, documented with the Tsview v.6.1.3.2 software (Tucsen Imaging Technology Co. Limited). In order to calculate secretion rate per area and mass unit, both leaf area (cm^2) and leaf dry weight (g) were measured. The leaf area was measured with the Area Meter LI-COR 3100 (Lincoln, USA), while the dry mass was obtained by weighing the samples after they were oven-dried at 60°C until constant weight.

2.3. *Root anatomy*

The thickness of epidermal, cortex and total root, air gap area and diameter of vascular cylinder of absorption roots were calculated. Roots were dehydrated using ethanol series, embedded in historesin (Leica, Germany), cross-sectioned (8–10 μm thick) using a rotary microtome, and stained with 0.05% toluidine blue, pH 4.7 (O'Brien et al., 1964). Thirty measurements per root section were performed using the software Nikon NIS-Elements (Tokyo, Japan). Pictures from analyzed sections were recorded using a light microscope (Nikon Eclipse 50i, Tokyo, Japan), equipped with a digital camera.

To compare differences between species, we considered the variance of data obtained from each treatment, which were compared to the corresponding control.

2.4. *Iron histochemistry staining of roots, stems and leaves*

Freehand sections from roots (*ca.* 2cm root apex), stems (*ca.* 10 cm from the shoot apex), and leaves (middle third) were obtained with a razorblade and immersed in Perl's reagent (1% potassium ferrocyanide, 2% HCl in a 1:1 proportion; Bancroft et al., 2008) for Fe(III) detection. Additionally, another set of plants sections were treated with Lillie's reagent (400 mg of potassium ferricyanide in 40 mL 0.5% HCl; Bancroft et al., 2008) for Fe(II) detection. After 24 hours, sections were washed; pictures from analyzed sections were recorded using a light microscope (Nikon Eclipse 50i, Tokyo, Japan), equipped with a digital camera. Positive staining was verified by the Prussian blue color of Perl's reagent (ferric iron), or by the Turnbull blue from Lillie's reagent (ferrous iron).

2.5. Statistical analysis

All data were tested for normal distribution. When normally distributed, we used the analysis of variance followed by the Tukey's test, and when not normally distributed, we used the Kruskal-Wallis test, both with a significance level $P < 0.05$.

The following multivariate statistical methods were applied to different sets of data (chemical, biological and combination parameters): canonical correlation analysis (CCA), Spearman's rank correlation coefficient and generalized Procrustes analysis (GPA). CCA and Spearman's rank were used to analyze correlations between two groups of variables (chemical and biological). Spearman's rank shows correlations between variables. CCA improves the information between iron bioaccumulation and biological responses. GPA was used with six dataset (chemical and biological data from three studied species) to understand their distribution and correlations.

Multivariate statistical methods are important to highlight the contribution of various variables in the model and their ability to discriminate one class from another (Wunderlin et

al., 2001). The statistical packages Statistica 12 and Infostat (Di Rienzo et al., 2010) were used for analyzes.

3. Results

3.1. Iron concentration in plaque, roots, stems, leaves and saline secretion from salt glands

Figure 1 and Tables 1S and 2S (supplementary material) show the iron concentration in plaque, roots, stems and leaves of three studied species under different concentrations of added-iron. Figure 2 and Table 1S and 2S show the iron concentration in secretions from salt glands. Controls show presence of iron due to the content of this element in the nutritive medium. These results evidence that the Fe-plaque is a place of high iron accumulation in these plants (Table 2S). *L. racemosa* accumulated the highest amounts of iron in the plaque and roots, while *A. schaueriana* presented the highest levels in stems and leaves (Figure 1 and Table 1S). Also in Figure 1, it is evident that the bioaccumulation of iron in the plaque and roots of *L. racemosa* increased progressively as the added-iron concentration rose. Figure 2 and Table 1S show that the iron secretion through salt glands is strongly inhibited in *L. racemosa* at iron concentrations above controls, while *A. schaueriana* did not show a noticeable drop from controls to the highest added-iron concentration used. On the other hand, salt glands density in both *A. schaueriana* and *L. racemosa* do not significantly vary throughout the different amounts of added-iron (Table 1S).

3.2. Translocation factors

Figure 3 and Table 1S show translocation factors between different parts of the studied plants, exposed to diverse added-iron amounts. A drop in Tf between aerial parts of *R. mangle* and its root (TFsr, TFtr and TFsh) becomes evident. Conversely, *R. mangle* showed a trend to increase Tf between stems and leaves, although not statistically significant (Figure 3, Table

1S). The rest of studied species did not show changes in Tf at all dosages of iron, although *A. schaueriana* had a significant drop in Tf when exposed to 100 mg L⁻¹ Fe(II). On the other hand, *L. racemosa* evidenced a drop in Tf only between leaves and stems, and between leaves and roots, at the maximum iron concentration used (Figure 3, Table 1S). In general, *L. racemosa* presented the lowest Tf among the three studied mangrove plants (Table 1S).

3.3. Root anatomy

Table 2 shows studied anatomical parameters for roots of the three studied species. Most analyzed parameters did not show significant changes throughout studied added-iron concentrations. The parameters showing changes were thickness of cortex and root in *A. schaueriana*, with higher values in controls compared to exposed plants. Additionally, the root of *L. racemosa* was significantly thinner in plants exposed to 100 mg L⁻¹ and thicker upon exposure to 20 mg L⁻¹ (Table 2).

3.4. Histochemistry

Figures 4, 5 and 6 show results from histochemical analysis.

From Figure 4 we can observe the main places of iron bioaccumulation in roots. Mainly the epidermis, but also cortex, were the areas showing the highest accumulation of both iron species (Fe(II) and Fe(III)) in the three studied species. Iron was also detected in vessel elements of *R. mangle* and in vascular cambium of *L. racemosa* exposed to the highest dose (100 mg L⁻¹).

Figure 5 shows that the vascular cambium of *L. racemosa* and the periderm of *R. mangle* were the main places for bioaccumulation of both iron species in stems from these two plant species. Conversely, in *A. schaueriana* it was not possible to verify the main places for iron bioaccumulation within the stem. However, stems of *A. schaueriana* showed presence

of Fe(II) and Fe(III) in epidermis, subepidermis, lenticel, xylem (mainly in protoxylem) and salt glands cells. Moreover, Fe(II) was massively detected in vessel elements, phloem, cortical parenchyma and pith of *A. schaueriana*.

Figure 6 shows some places of iron bioaccumulation in leaves. The main place for bioaccumulation in *A. schaueriana* was the water storage parenchyma and, additionally, the cortex of the midvein, epidermis, salt glands and spongy parenchyma. On the other hand, *L. racemosa* presented the main iron bioaccumulation in its xylem, but also lower bioaccumulation in salt glands, and in both water storage and palisade parenchyma. Finally, the bioaccumulation of iron in leaves of *R. mangle* occurred in several places, mainly in the cortex of the midvein, and in both palisade and water storage parenchyma.

3.5. Statistical analysis

Chemical data (Table 1S) and anatomical information (Table 2) were independently evaluated for each plant species to assess the plant that best correlates the bioaccumulation with changes in the anatomy. CCA demonstrates that *L. racemosa* was the species showing the best correlation between chemical and biological matrix ($R^2 = 0.98$, $P = 0.01$), followed by *A. schaueriana* ($R^2 = 0.97$, $P = 0.03$), with no significant correlation for *R. mangle* ($R^2 = 0.89$, $P = 0.3$).

Spearman test showed a positive correlation between the iron concentration in the Fe-plaque and the amount of iron in roots of *L. racemosa* (Spearman $R = 0.80$) and *R. mangle* (Spearman $R = 0.82$). In addition, a negative correlation was found between iron concentration in plaque and translocation factors to *R. mangle* (Spearman R for $TF_{sr} = -0.77$, $TF_{lr} = -0.84$, $TF_{sh} = -0.84$) and *L. racemosa* (Spearman R for $TF_{lr} = -0.64$). No significant correlation ($P > 0.05$) was found between the density of salt glands and the concentration of iron in the secretion from *A. schaueriana* and *L. racemosa*. Furthermore, no significant

correlation was found between the iron concentration from salt glands secretion and the amount of iron in leaves of *A. schaueriana* and *L. racemosa*.

A GPA was performed to evidence the correspondence between chemical parameters (Table 1S) and biological data (Table 2) along the three studied mangrove plants. Figure 7 shows the graphical representation of GPA, with the first function (CP1) explaining 53.5% of the total variance, and 27.0% for the second function (CP2). Also from Figure 7 it is evident that four studied groups (corresponding to different levels of added-iron) can be clearly separated by considering the three studied mangrove species, mainly when exposed to the highest iron level (100 mg L⁻¹).

4. Discussion

4.1. Bioaccumulation of iron from exposure medium to different plant compartments

Sections 3.1 and 3.2 describe results found for the uptake, translocation and bioaccumulation of iron from the exposure medium to different plant compartments, including secretion through salt glands present in leaves of two species. From Figures 1A and Table 1S, it is evident that *L. racemosa* showed a dose-dependent behavior, with low levels of iron in both plaque and roots in controls, and a progressive increase until reaching maximum values at the highest added-iron concentration. Also from Figure 1A, we can observe that *R. mangle* presented a similar behavior to *L. racemosa* with a less pronounced response to increased amounts of added-iron. So far, roots of *L. racemosa* could be proposed as good bioindicator for iron in the mangrove environment.

Reports from the literature point out that compounds rich in iron oxides and hydroxides are attached to the root in wetland plants (Tripathi et al., 2014). Thus, the Fe-plaque could act as a buffer (Jiang et al., 2009; Xu et al., 2009), or as a barrier regulating the uptake of metals by the plant (Cheng et al., 2014; Pan et al., 2014). Our current results with *L.*

racemosa and *R. mangle* (Table 1S and Spearman statistical analysis) show a positive correlation between the amount of iron in the plaque and the corresponding level in the root (internal). Thus, it is possible to believe that the plaque is not limiting the input of iron from the environment to the root, but probably acting as a buffer.

From Figure 1B, we observed that stems and leaves did not show a clear dose-dependent response, with the exception of stems from *A. schaueriana* and *L. racemosa*, which evidenced a significant increase of iron in stems of plants exposed to the highest concentration compared to controls.

We did not observe higher iron content in roots when compared to aerial parts of *A. schaueriana*. That result do not agree with reports from the literature on wild species exposed to environmental iron (*e.g.* Souza et al. 2015). The large bioaccumulation of iron found during this work in leaves of studied species reinforce the importance of this element for the structure of chlorophyll and photosynthesis (Briat et al., 2015). Furthermore, reports on the high accumulation of iron and other metals in whole-root (plaque + internal) could be due to the retention of metals in the Fe-plaque, and not to a real bioaccumulation of these elements within the root (internal).

Differences found in the bioaccumulation of iron in roots, stems and leaves of the three studied species could be attributed to the regulatory mechanisms of salt in roots, which allows the entry of other metals in the process (Lacerda et al., 1985). Generally, salt secreting plants have higher concentrations of salt in the xylematic sap than those without salt-secreting glands (Scholander et al., 1962). In these cases, the salt ultra-filtration mechanism present in roots is responsible for this difference. In *R. mangle*, a species without salt glands, both external hypodermis and endodermis seem to work as two sheaths of the ultrafilter, which would explain the lower concentration of salt found in the sap (Werner and Stelzer, 1990). In this context, some studies have shown that metals concentrations, such as iron and other

nutrients, are higher in leaves of salt-secreting plants when compared to species that do not secrete salt (Bernini et al., 2006; Cuzzuol and Campos, 2001; Sarangi et al., 2002). Higher concentrations of iron in leaves have been reported in *Avicennia* and *Laguncularia*, compared to the levels observed in *Rhizophora* (Bernini et al., 2006; Cuzzuol and Campos, 2001). Previous and current results from our research group demonstrate a preferential bioaccumulation of iron (and other elements) in leaves of *A. schaueriana* and *L. racemosa* in relation to *R. mangle*, corroborating this trend (Souza et al., 2014a, 2014b; 2015).

Figure 2 shows that the secretion of iron from salt glands of *L. racemosa* is strongly inhibited upon exposure to added-iron, while *A. schaueriana* did not exhibit a clear change, despite showing significant drops in relation to controls at both 10 and 100 mg L⁻¹ added-iron. So far, and considering this differential behavior between the studied plants when exposed to increasing Fe(II) concentrations, we could propose *L. racemosa* as the best bioindicator species for biomonitoring of iron in mangrove areas under risk of iron pollution. Moreover, the use of salt glands secretion from *L. racemosa* can be proposed as a method for sensing iron pollution without excessively disturbing the plant.

We observe similarities when comparing our current results with previous reports on the presence and distribution of metals in wild mangrove plants, using the same species sampled from the study area where propagules for this study were obtained (Vitoria, Espírito Santo, Brazil). For instance, wild *A. schaueriana* showed a variable response between levels of iron in roots in relation to bioavailable iron in the environment (Souza et al., 2015); a similar behavior was reported for wild *R. mangle* (Souza et al., 2014a) and for wild *L. racemosa* (Souza et al., 2014b). These reports indicate that the sum of internal and external levels of iron in roots of wild *L. racemosa* ranged from 3,995 to 7,169 µg g⁻¹ (average= 4,864 µg g⁻¹; Souza et al., 2014b); in *R. mangle*, levels ranged from 804 to 13,477 µg g⁻¹ (average= 4,993 µg g⁻¹; Souza et al., 2014a); and in *A. schaueriana*, levels ranged from 1,319 to 8,938

$\mu\text{g g}^{-1}$ (average= 3,205 $\mu\text{g g}^{-1}$; Souza et al., 2015). So far, *L. racemosa* and *R. mangle* showed the highest average bioaccumulations of iron in roots, in agreement with our current results, although previous reports did not differentiate between Fe-plaque and root (external and internal iron in the root).

Furthermore, when comparing our current results for iron in leaves with field reports, we can observe that wild *L. racemosa* ranged from 107 to 427 $\mu\text{g g}^{-1}$ (average= 211 $\mu\text{g g}^{-1}$; Souza et al., 2014b); *R. mangle* ranged from 71 to 183 $\mu\text{g g}^{-1}$ (average= 101 $\mu\text{g g}^{-1}$; Souza et al., 2014a); and *A. schaueriana* ranged from 97 to 665 $\mu\text{g g}^{-1}$ (average= 308 $\mu\text{g g}^{-1}$; Souza et al., 2015). Thus, results for iron in leaves from the three studied species also show good agreement between field and experimental exposure.

There are not previous reports on levels of iron in stems or in secretions from salt glands of studied plants evaluated in the field. To our knowledge, this is the first report evaluating secreted iron using increasing levels of Fe(II) during the growth of mangrove plants. It is worth to remark that controlled studies could help evaluate the behavior of a particular element, isolating it from the complex matrix associated with environmental samples.

4.2. Translocation of iron from root to aerial plant compartments

Differences observed with translocation factors (Tf) were not so evident as in the results obtained with bioaccumulation. However, in general, increasing dosages of iron decreases the translocation of this metal into the plants (Table 1S, Figure 3). A significant drop of translocation was found in *R. mangle* and *A. schaueriana* when evaluating aerial parts and roots (TFsr, TF_{lr} and TFsh) (Figure 3, Table 1S). This drop was dose-dependent. *L. racemosa* did not show changes in TFsr and TFsh throughout the studied concentrations of

added-iron. This could evidence that higher concentrations of iron in the substrate could more negatively affect Tf in *R. mangle* and *A. schaueriana* than in *L. racemosa*.

The statistical analysis showed a negative correlation (Spearman) between Tf and iron concentration in the plaque for *L. racemosa* and *R. mangle*. Some studies have demonstrated that the Fe-plaque can diminish the translocation of some metals from the root to aerial parts of the plant (Cheng et al., 2014; Liu et al., 2016). Thus, our current results agree with these reports.

4.3. Anatomical and histochemical changes in exposed plants

Few anatomic alterations were found in roots of plants exposed to increasing levels of iron (Table 2). No correlation was observed between the Fe-plaque and the size of air gap. So far, an incidence of the aerenchymal area on the Fe-plaque in the root could be not expected. This last result does not agree with the report of Pi et al. (2010), on the effects of wastewater in two mangrove plants. They observed a greater area of air gap, with formation of more Fe-plaque, due to more radial oxygen loss (ROL). Deng et al. (2010) also noted the positive correlation between the aerenchymal area and formation of Fe-plaque, although they found that it was not due to the increased release of ROL, but to the root surface area for Fe-plaque grip.

On the other hand, the lower root thickness observed when the concentration of added-iron rose confirms some previous reports. For instance, Cheng et al. (2012) observed a decrease in total biomass in six out of eight mangrove plants exposed to iron. On the other hand, Alongi (2010) found an increase in total plant biomass, but a reduction in root growth in two out of five mangrove species studied.

Plant epidermis was confirmed as the main compartment for iron bioaccumulation in roots (Figure 4), probably being the main barrier to the uptake of metals by the plant. However, histochemical analysis did not detect bioaccumulation of iron in the root endoderm.

In this study, the detection of iron by histochemical test in walls and cell content of leaves, roots and stems, suggests that there is a short-distance for the transport of iron via both apoplast and symplast, as reported by Bauer and Hell (2006). The release of iron in xylem vessels requires the efflux of Fe from the symplast to the apoplastic space. It is generally accepted that iron is present forming Fe(III)-citrate complexes in the xylem. However, the mechanism involved in the absorption of iron in xylem vessels and leaf tissues is still unclear (Kim and Guerinot, 2007).

The secretion of iron through salt glands in *A. schaueriana* and *L. racemosa* was first tested using histochemistry (Figure 6), and further confirmed by chemical analysis (ICP-MS) (Table 1S, Figure 2). Our current results do not show an increase in secreted iron as added-iron concentrations rose. Conversely, *L. racemosa* showed a significant drop, while *A. schaueriana* presented a non-clear pattern with a significant drop when comparing controls with the higher iron-dose. These results do not agree with previous reports by Macfarlane and Burchett (1999, 2000) and Naidoo et al. (2014), who used increasing amounts of zinc and Cu in sediments, resulting in higher Zn and Cu elimination through salt glands. This contradictory result could indicate that the secretion of iron through salt glands could be interfered by other factors. Particularly, *L. racemosa* seems to be more sensitive to changes in the iron concentration. This could not be attributed to the drop in Tf at higher added-iron concentrations, mainly because levels of iron in leaves of *L. racemosa* remained almost unchanged (Figure 1B, Table 1S). Moreover, no correlation was found between the amount of secreted iron and the density of salt glands in leaves of *L. racemosa* or in *A. schaueriana*.

(Table 1S). Further research is needed to elucidate the inhibition observed in secreted iron. To our knowledge, this is the first report on salt glands in stems of *A. schaueriana* (Figure 5).

5. Conclusions

Three studied plants are capable of biosensing the amount of added-Fe(II). *L. racemosa* showed a dose-dependent response in root and plaque, in addition to an inhibitory behavior with secretion of iron through salt glands. So far, we propose *L. racemosa* as the most appropriate species for biosensing the amount of iron in mangroves exposed to high amounts of this element due to environmental pollution. Furthermore, the inhibition of iron secretion through salt glands enables the use of this parameter for environmental biomonitoring, avoiding excessive damage on wild plants, although bioaccumulation in the root of this species (both internal and plaque) presented the best response.

A drop in translocation factors between aerial parts of the plant and the root were evident and significant, mainly in *R. mangle* and *A. schaueriana*, indicating the impact on the plant transportation mechanism induced by high concentrations of added-Fe(II).

Changes in plants anatomy and histochemistry were not so evident as those observed with bioaccumulation and translocation.

To our knowledge, this is the first detailed report on the response of these plants to increasing iron concentrations under controlled conditions, complementing existing data on the behavior of the same plants under field exposure.

6. Acknowledgements

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References

- Alongi, D.M., 2010. Dissolved iron supply limits early growth of estuarine mangroves. *Ecology* 91, 3229–3241.
- Arrivabene, H.P., Souza, I.C, C6, W.L.O., Conti, M.M., Wunderlin, D.A., Milanez, C.R.D, 2015. Effect of pollution by particulate iron on the morphoanatomy, histochemistry, and bioaccumulation of three mangrove plant species in Brazil. *Chemosphere* 127, 27–34.
- Bancroft, J.D., Stevens, A., Turner, D.R. Theory and practice of histological techniques. New York: Churchill Livingstone; 2008.
- Barbier, E.B., Hacker, S.D., Kennedy, C., Koch, E.W., Stier, A.C., Silliman, B.R, 2011. The value of estuarine and coastal ecosystem services. *Ecol. Monogr.* 81, 169–193.
- Bauer, P., Hell, R., 2006. Translocation of iron in plant tissues. In: Barton, L.L., Abadia, J., editors. Iron nutrition in plants and rhizospheric microorganisms. Dordrecht: Springer. p. 279–288.
- Bernini, E., Silva, M.A.B., Carmo, T.M.S., Cuzzuol, G.R.F, 2006. Composi7o qumica do sedimento e de folhas das espcies do manguezal do esturio do Rio So Mateus, Esprito Santo, Brasil. *Revista Brasil. Bot.* 29, 689–699.
- Brander, L.M., Wagtendonk, A.J., Hussain, S.S., McVittie, A., Verburg, P.H., de Groot, R.S., Van der Ploeg, S., 2012. Ecosystem service values for mangroves in Southeast Asia: A meta-analysis and value transfer application. *Ecosystem Services* 1, 62–69.
- Briat, J.F., Dubos, C., Gaymard, F., 2015. Iron nutrition, biomass production, and plant product quality. *Trends Plant Sci.* 20, 33–40.
- Cardwell, R.D., Deforest, D.K., Brix, K.V., Adams, W.J., 2013. Do Cd, Cu, Ni, Pb, and Zn biomagnify in aquatic ecosystems?. *Rev. Environ. Contam. T.* 226, 101–122.

- Chappaz, A., Lyons, T.W., Gordon, G.W., Anbar, A.D., 2012. Isotopic fingerprints of anthropogenic molybdenum in lake sediments. *Environ. Sci. Technol.* 46, 10934–10940.
- Cheng, H., Chen, D.T., Tam, N.F.Y., Chen, G.Z., Li, S.Y., Ye, Z.H., 2012. Interactions among Fe^{2+} , S^{2-} , and Zn^{2+} tolerance, root anatomy, and radial oxygen loss in mangrove plants. *J. Exp. Bot.* 63, 2619–2630.
- Cheng, H., Wang, M., Wong, M.H., YE, Z., 2014. Does radial oxygen loss and iron plaque formation on roots alter Cd and Pb uptake and distribution in rice plant tissues?. *Plant Soil* 375, 137–148.
- Clough, B. Continuing the journey amongst mangroves. ISME Mangrove Educational Book Series No. 1. International Society for Mangrove Ecosystems (ISME), Okinawa, Japan, and International Tropical Timber Organization (ITTO). Japan: Yokohama; 2013.
- Costanza, R., D'Arge, R., Groot, R.D., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., Belt, M.V.D., 1997. The value of the world's ecosystem services and natural capital. *Nature* 387, 253–260.
- Cuzzuol, G.R.F., Campos, A., 2001. Aspectos nutricionais na vegetação de manguezal do estuário do Rio Mucuri, Bahia, Brasil. *Revista Brasil. Bot.* 24, 227–234.
- Defew, L.H., Mair, J.M., Guzman, H.M., 2005. An assessment of metal contamination in mangrove sediments and leaves from Punta Mala Bay, Pacific Panama. *Mar. Pollut. Bull.* 50, 547–552.
- Deng, D., Wu, S.C., Wu, F.Y., Deng, H., Wong, M.H., 2010. Effects of root anatomy and Fe plaque on arsenic uptake by rice seedlings grown in solution culture. *Environ. Pollut.* 158, 2589–2595.

- Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., Robledo, C.W. InfoStat Versión. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina; 2010.
- Du, J., Yan, C., Li, Z., 2013. Formation of iron plaque on mangrove *Kandalar. Obovata (SL)* root surfaces and its role in cadmium uptake and translocation. Mar. Pollut. Bull. 74, 105–109.
- Fitzgerald, M.A., Allaway, W.G., 1991. Apoplastic and symplastic pathways in the leaf of the grey mangrove *Avicennia marina* (Forsk.) Vierh. New Phytol. 119, 217–226.
- Giri, C., Ochieng, E., Tieszen, L.L., Zhu, Z., Singh, A., Loveland, T., Masek, J., Duke, N., 2011. Status and distribution of mangrove forests of the world using earth observation satellite data. Global Ecol. Biogeogr. 20, 154–159.
- Hoagland, D.R., Arnon, D.I. The water culture method for growing plants without soils. Berkeley: California Agricultural Experimental Station; 1950.
- Instituto Brasileiro de Executivos de Finanças – IBEF. Disponível em: <<http://ibefes.ning.com/page/ibefes-faz-visita-tecnica-a>>. Acesso em: 13 fev. 2011.
- Jiang, F.Y., Chen, X., Luo, A.C., 2009. Iron plaque formation on wetland plants and its influence on phosphorus, calcium and metal uptake. Aquat. Ecol. 43, 879–890.
- Kim, S.A., Guerinot, M.L., 2007. Mining iron: iron uptake and transport in plants. FEBS Lett. 581, 2273–2280.
- Kobayashi, T., Nishizawa, N.K., 2012. Iron uptake, translocation, and regulation in higher plants. Annu. Rev. Plant Biol. 63, 131–152.
- Lacerda, L.D., Rezende, C.E., José, D.V., Wasserman, J.C., Francisco, M.C., 1985. Mineral concentrations in leaves of mangrove trees. Biotropica 17, 21–27.

- Lacerda, L.D., Souza, C.M.M., Pestana, M.H.D., 1989. Trace metals geochemical associations in sediments of a non-contaminated estuary. *Ciência e Cultura* 41, 301–304.
- Liu, C.Y., Gong, X.F., Chen, C.L., Yang, J.Y., Xu, S., 2016. The effect of iron plaque on lead translocation in soil- *Carex cinerascens* Kukenth. system. *Int. J. Phytoremediation* 18, 1–9.
- Liu, W.J., Zhu, Y.G., Smith, F.A., Smith, S.E., 2004. Do iron plaque and genotypes affect arsenate uptake and translocation by rice seedlings (*Oryza sativa* L.) grown in solution culture?. *J. Exp. Bot.* 55, 1707–1713.
- Lu, H., Liu, B., Zhang, Y., Ye, J.; Yan, C., 2014. Comparing analysis of elements sub-cellular distribution in *Kandelia obovata* between SEM-EDX and chemical extraction. *Aquat. Bot.* 112, 10–15.
- MacFarlane, G.R., Burchett, M.D., 1999. Zinc distribution and excretion in the leaves of the grey mangrove, *Avicennia marina* (Forsk.) Vierh. *Environ. Exp. Bot.* 41, 167–175.
- MacFarlane, G.R., Burchett, M.D., 2000. Cellular distribution of copper, lead and zinc in the grey mangrove, *Avicennia marina* (Forsk.) Vierh. *Aquat. Bot.* 68, 45–59.
- Menezes, N.L.D., 2006. Rhizophores in *Rhizophora mangle* L: an alternative interpretation of so-called "aerial roots". *An. Acad. Bras. Cienc.* 78, 213–226.
- Morse, J.W., Rickard, D., 2004. Peer reviewed: chemical dynamics of sedimentary acid volatile sulfide. *Environ. Sci. Technol.* 38, 131A–136A.
- Naidoo, G., Hiralal, T., Naidoo, Y., 2014. Ecophysiological responses of the mangrove *Avicennia marina* to trace metal contamination. *Flora* 209, 63–72.
- Nath, B., Chaudhuri, P., Birch, G., 2014. Assessment of biotic response to heavy metal contamination in *Avicennia marina* mangrove ecosystems in Sydney Estuary, Australia. *Ecotox. Environ. Safe.* 107, 284–290.

- Nica, D.V., Bura, M., Gergen, I., Harmanescu, M., Bordean, D.M., 2012. Bioaccumulative and conchological assessment of heavy metal transfer in a soil-plant-snail food chain. *Chem. Cent. J* 6, 1–15.
- O'Brien, T.P., Feder, N., McCully, M.E., 1964. Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma* 59, 368–73.
- Otte, M.L., Dekkers, I.M.J., Rozema, J., Broekman, R.A., 1991. Uptake of arsenic by *Aster tripolium* in relation to rhizosphere oxidation. *Can. J. Bot.* 69, 2670–2677.
- Pan, W., Wu, C., Xue, S., Hartley, W., 2014. Arsenic dynamics in the rhizosphere and its sequestration on rice roots as affected by root oxidation. *J. Environ. Sci.* 26, 892–899.
- Pi, N., Tam, N.F.Y., Wong, M.H., 2010. Effects of wastewater discharge on formation of Fe plaque on root surface and radial oxygen loss of mangrove roots. *Environ. Pollut.* 158, 381–387.
- Pi, N., Tam, N.F.Y., Wong, M.H., 2011. Formation of iron plaque on mangrove roots receiving wastewater and its role in immobilization of wastewater-borne pollutants. *Mar. Pollut. Bull.* 63, 402–411.
- Sadiq, M., Zaidi, T.H., 1994. Sediment composition and metal concentrations in mangrove leaves from the Saudi coast of the Arabian Gulf. *Sci. Total Environ.* 155, 1–8.
- Sarangi, R.K., Kathiresan, K., Subramanian, A.N., 2002. Metal concentrations in five mangrove species of the Bhitarkanika, Orissa, east coast of India. *Indian J. Mar. Sci.* 31, 251–253.
- Scholander, P.F., Hammel, H.T., Hemmingson, E.A., Garey, W., 1962. Salt balance in mangroves. *Plant Physiol.* 37, 722–729.
- Sherman, R.E., Fahey, T.J., Howarth, R.W., 1998. Soil-plant interactions in a neotropical mangrove forest: iron, phosphorus and sulfur dynamics. *Oecologia* 115, 553–563.

- Silva, C.A.R., Lacerda, L.D., Rezende, C.E., 1990. Metals reservoir in a red mangrove forest. *Biotropica* 22, 339–345.
- Souza, I., Bonomo, M. M., Morozesk, M., Rocha, L. D., Duarte, I. D., Furlan, L. M., Arrivabene, H.P., Monferrán, M.V., Matsumoto, S.T., Milanez, C.R.D., Wunderlin, D.A., Fernandes, M. N., 2014b. Adaptive plasticity of *Laguncularia racemosa* in response to different environmental conditions: integrating chemical and biological data by chemometrics. *Ecotoxicology* 23, 335–348.
- Souza, I.C., Morozesk, M., Duarte, I.D., Bonomo, M.M., Rocha, L.D., Furlan, L.M., Arrivabene, H.P., Monferrán, M.V., Matsumoto, S.T., Milanez, C.R.D., Wunderlin, D.A., Fernandes, M.N., 2014a. Matching pollution with adaptive changes in mangrove plants by multivariate statistics. A case study, *Rhizophora mangle* from four neotropical mangroves in Brazil. *Chemosphere* 108, 115–124.
- Souza, I.C., Rocha, L.D., Morozesk, M., Bonomo, M.M., Arrivabene, H.P., Duarte, I.D., Furlan, L.M., Monferrán, M.V., Matsumoto, S.T., Milanez, C.R.D., Wunderlin, D.A., Fernandes, M.N., 2015. Changes in bioaccumulation and translocation patterns between root and leaves of *Avicennia schaueriana* as adaptive response to different levels of metals in mangrove system. *Mar. Pollut. Bull.* 94, 176–184.
- Taylor, G.J., Crowder, A.A., 1983. Use of the DCB technique for extraction of hydrous oxides from roots of wetlands plants. *Am. J. Bot.* 70, 1254–1257.
- Tomlinson, P.B. The botany of mangroves. New York: Cambridge University Press; 1994.
- Tripathi, R.D., Tripathi, P., Dwivedi, S., Kumar, A., Mishra, A., Chauhan, P.S., Norton, G.J., Nautiyal, C.S., 2014. Roles for root iron plaque in sequestration and uptake of heavy metals and metalloids in aquatic and wetland plants. *Metallomics* 6, 1789–1800.
- Usepa. Risk-Based concentration table. Philadelphia PA. Washington: United States Environmental Protection Agency; 2009.

- Valiela, I., Bowen, J.L., York, J.K., 2001. Mangrove forests: One of the world's threatened major tropical environments. *Bioscience* 51, 807–815.
- Veerasingham, S., Vethamony, P., Murali, R. M., Fernandes, B., 2015. Depositional record of trace metals and degree of contamination in core sediments from the Mandovi estuarine mangrove ecosystem, west coast of India. *Mar. Pollut. Bull.* 91, 362–367.
- Vo, Q.T., Kuenzer, C., Vo, Q.M., Moder, F., Oppelt, N., 2012. Review of valuation methods for mangrove ecosystem services. *Ecol. Indic.* 23, 431–446.
- Werner, A., Stelzer, R., 1990. Physiological responses of the mangrove *Rhizophora mangle* grown in the absence and presence of NaCl. *Plant Cell Environ.* 13, 243–255.
- Wunderlin, D.A., Díaz, M.P., Amé, M.V., Pesce, S.F., Hued, A.C., Bistoni, M.A., 2001. Pattern recognition techniques for the evaluation of spatial and temporal variations in water quality. A case study: Suquía River Basin (Córdoba, Argentina). *Water Res.* 35, 2881–2894.
- Xu, D., Xu, J., He, Y., Huang, P.M., 2009. Effect of iron plaque formation on phosphorus accumulation and availability in the rhizosphere of wetland plants. *Water Air Soil Pollut.* 200, 79–87.
- Youssef, T., Saenger, P., 1996. Anatomical adaptive strategies to flooding and rhizosphere oxidation in mangrove seedlings. *Aust. J. Bot.* 44, 297–313.
- Zhou, Y.W., Zhao, B., Peng, Y.S., Chen, G.Z., 2010. Influence of mangrove reforestation on heavy metal accumulation and speciation in intertidal sediments. *Mar. Pollut. Bull.* 60, 1319–1324.

FIGURE CAPTIONS

Fig.1. Iron concentration ($\mu\text{g g}^{-1}$ dry weight) in (a) Fe-plaque and roots, and (b) stems and leaves of *A. schaueriana*, *L. racemosa* and *R. mangle* experimentally exposed to increasing amounts of added-Fe(II). Reported values correspond to the median. Significant differences for the same parameter, analyzed between different exposure concentrations for each plant, are pointed out using different letters (Kruskal-Wallis test, $P < 0.05$).

Fig.2. Iron concentration ($\mu\text{g g}^{-1}$ dry weight) in salt glands secretion of *A. schaueriana* and *L. racemosa* experimentally exposed to increasing amounts of added-Fe(II). Reported values correspond to the median. Significant differences, analyzed between different exposure concentrations for each plant, are pointed out using different letters (Kruskal-Wallis test, $P < 0.05$).

Fig.3. Translocation factors (Tfs) between leaves and roots, and aerial parts and roots of *A. schaueriana*, *L. racemosa* and *R. mangle* experimentally exposed to increasing amounts of added-Fe(II). Reported values correspond to the median. Significant differences for the same parameter, analyzed between different exposure concentrations for each plant, are pointed out using different letters (Kruskal-Wallis test, $P < 0.05$).

Fig. 4. Histochemical detection of Fe(II) or Fe(III) in cross-sections of roots. The blue color indicates a positive reaction to the test. (A-B) Fe(III) in *A. schaueriana* exposed to 10 (A) and 20 mg L^{-1} (B) of iron. (C-D) Fe(III) in *L. racemosa* exposed to 0 (C) and 10 mg L^{-1} (D) of added-iron. (E-F) Fe(II) in *R. mangle* exposed to 0 (E) and 20 mg L^{-1} (F) of added-iron.

(Arrow = epidermis, co = cortex, en = endodermis, pe = pericycle). Bars: (A)= 50 μm , (B)= 25 μm , (C, D, E, F) = 100 μm .

Fig. 5. Histochemical detection of Fe(II) or Fe(III) in cross-sections of stems. The blue color indicates a positive reaction to the test. (A-D) Fe(II) in *A. schaueriana* exposed to 10 (A, C, D) and 20 mg L^{-1} (B) of added-Fe(II). (E-F) *L. racemosa* exposed to 100 mg L^{-1} of added-Fe(II). Detection of Fe(II) (E) and Fe(III) (F). (G-H) *R. mangle* exposed to 100 mg L^{-1} of added-Fe(II). Detection of Fe(II) (G) and Fe(III) (H). (Arrow = epidermis, co = cortex, en = endodermis, pd = periderm, ph = phloem, pr = phloem ray; sg = salt gland, vc = vascular cambium, xy = xylem, xr = xylem ray). Bars: (A)= 50 μm , (B)= 25 μm , (C, D, E, F) = 100 μm .

Fig. 6. Histochemical detection of Fe(II) or Fe(III) in cross-sections of leaves. The blue color indicates a positive reaction to the test. (A-B) Fe (III) in *A. schaueriana* exposed to 20 mg L^{-1} of added-Fe(II). (C-D) Fe(III) in *L. racemosa* exposed to 10 (C) and 20 mg L^{-1} (D) of added-Fe(II). (E-F) *R. mangle* exposed to 10 mg L^{-1} of added-Fe(II). Detection of Fe(III) (E) and Fe(II) (F). (co = cortex, ph = phloem, sg = salt gland, sp = spongy parenchyma, wp = water-storage parenchyma, xy = xylem). Bars (C, F)= 100 μm , (A, D, E)= 50 μm and (B)= 25 μm .

Fig. 7. Generalized Procrustes Analysis (GPA) of parameters studied for each treatment with added-Fe(II). (AS = *A. schaueriana*, LR = *L. racemosa*, RM = *R. mangle*).

FIGURES

Figure 1

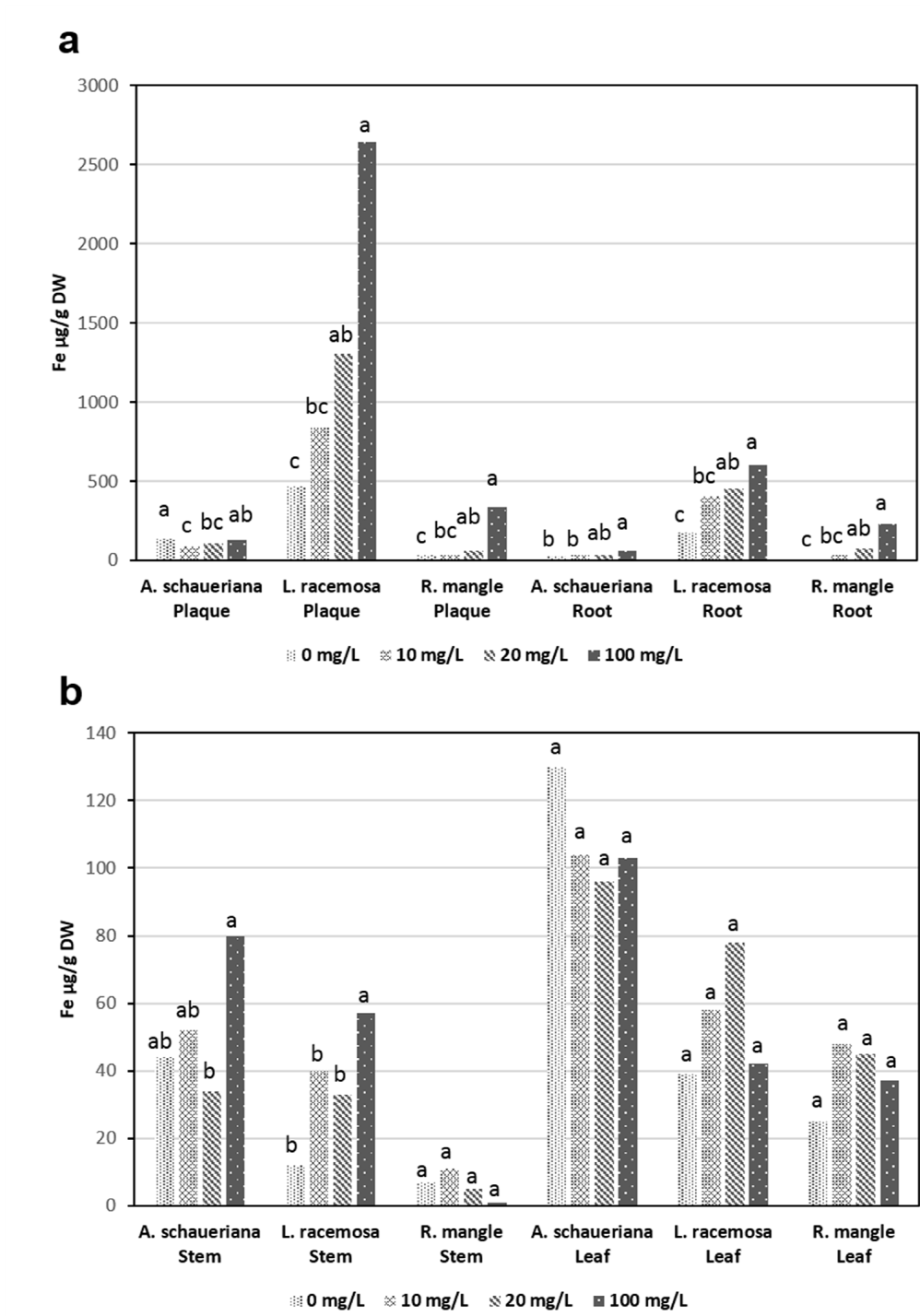


Figure 2

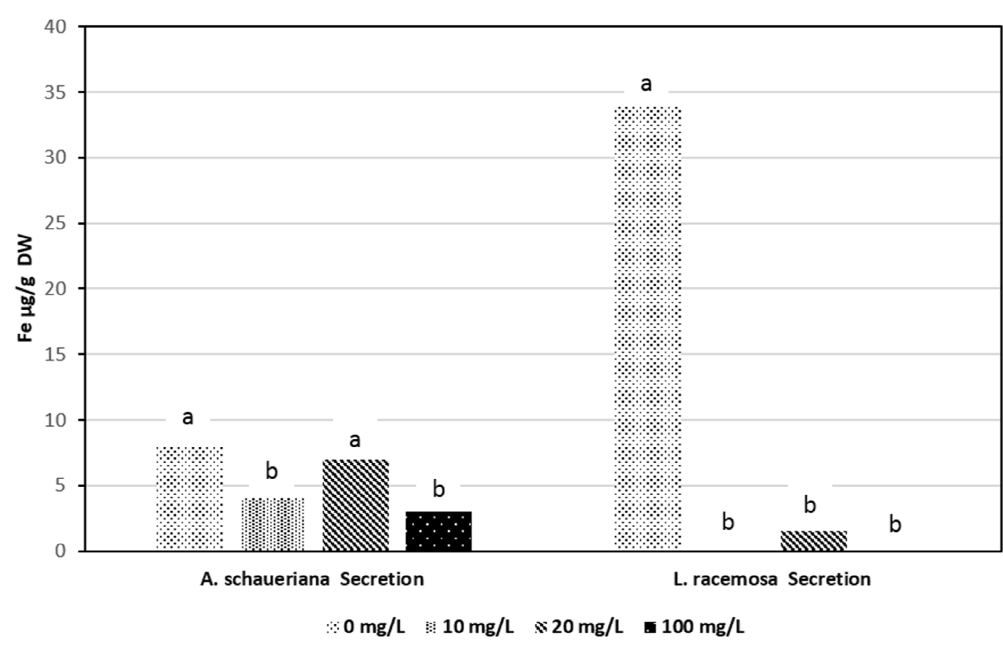


Figure 3

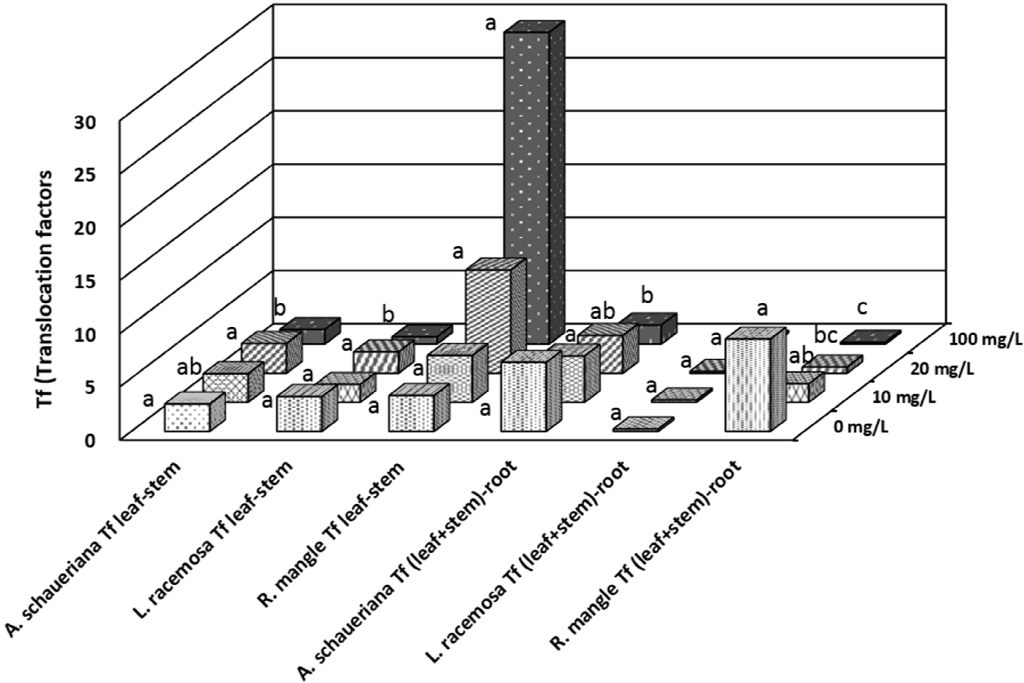


Figure 4

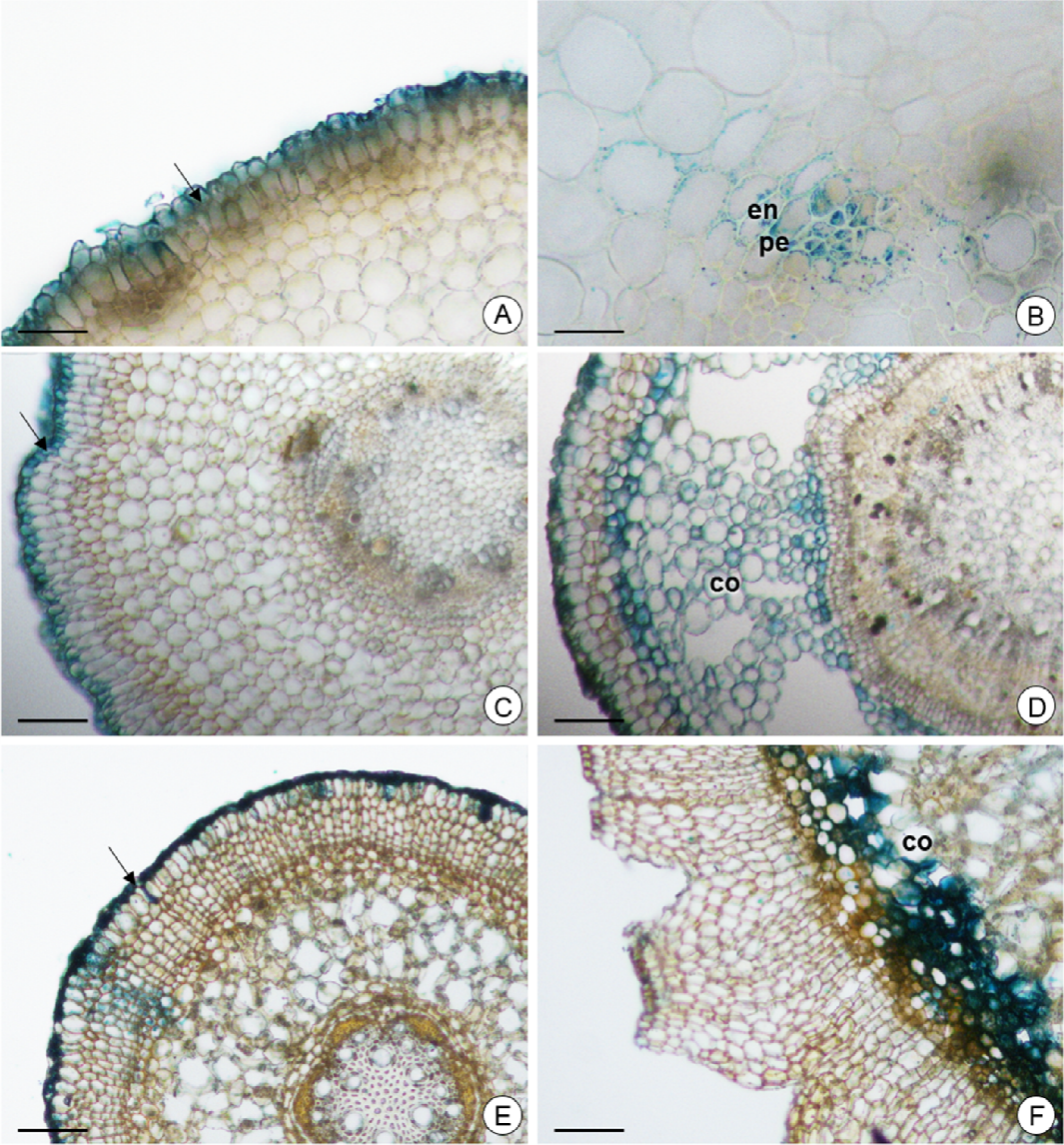


Figure 5

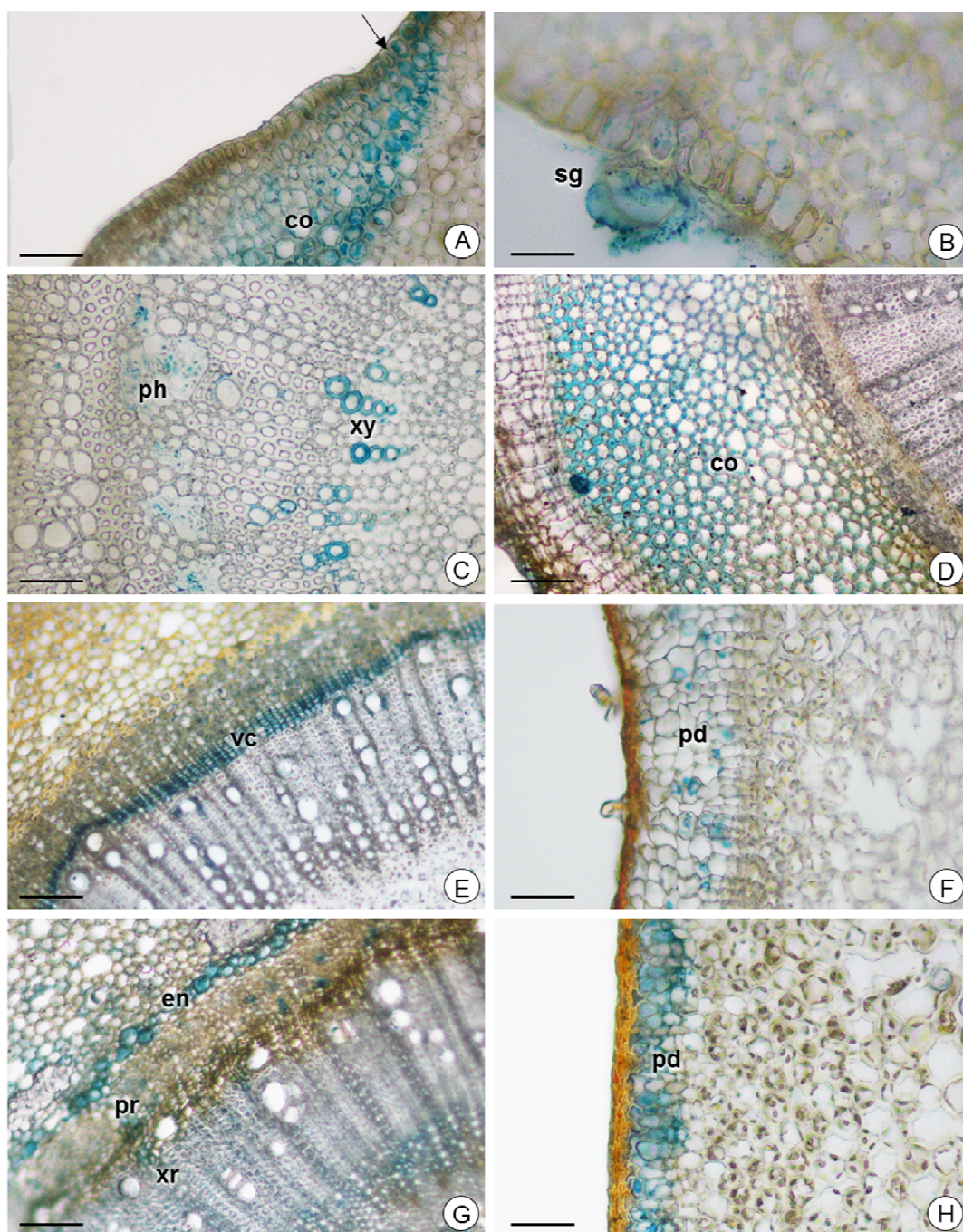


Figure 6

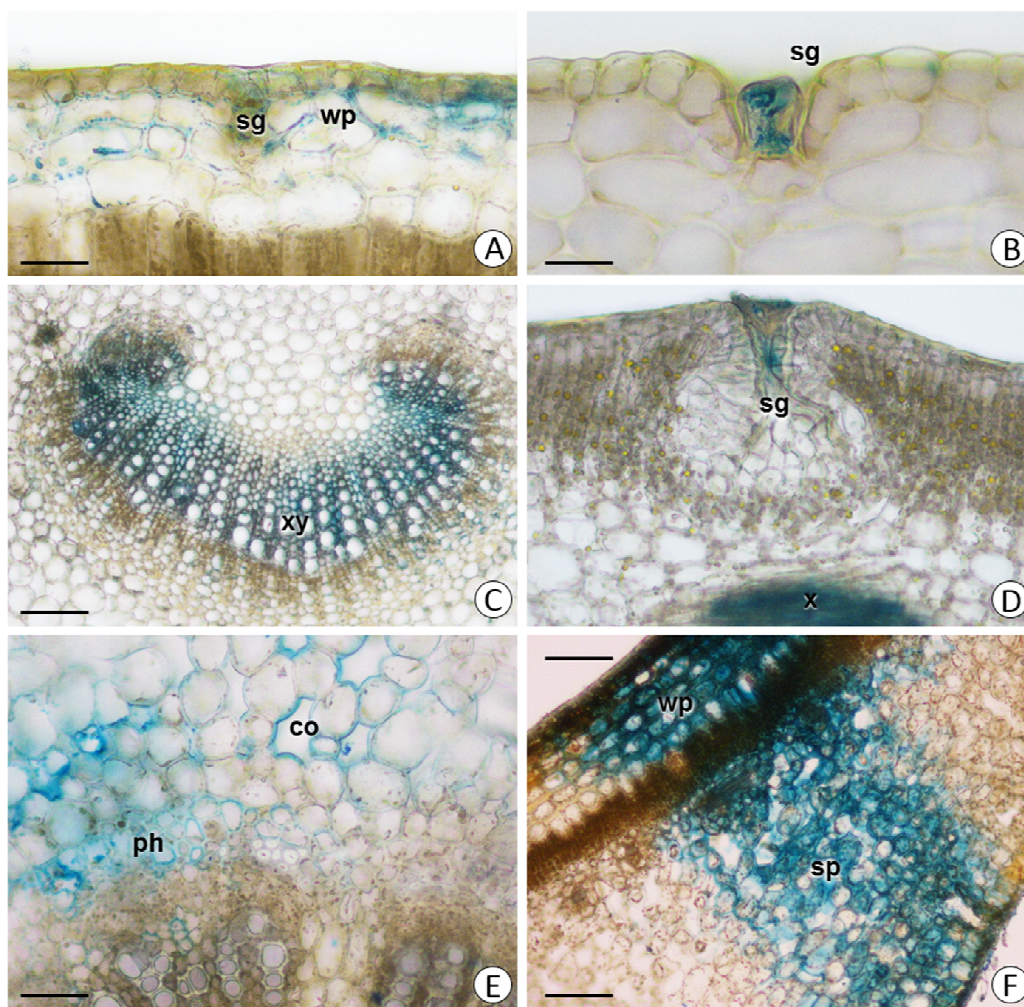
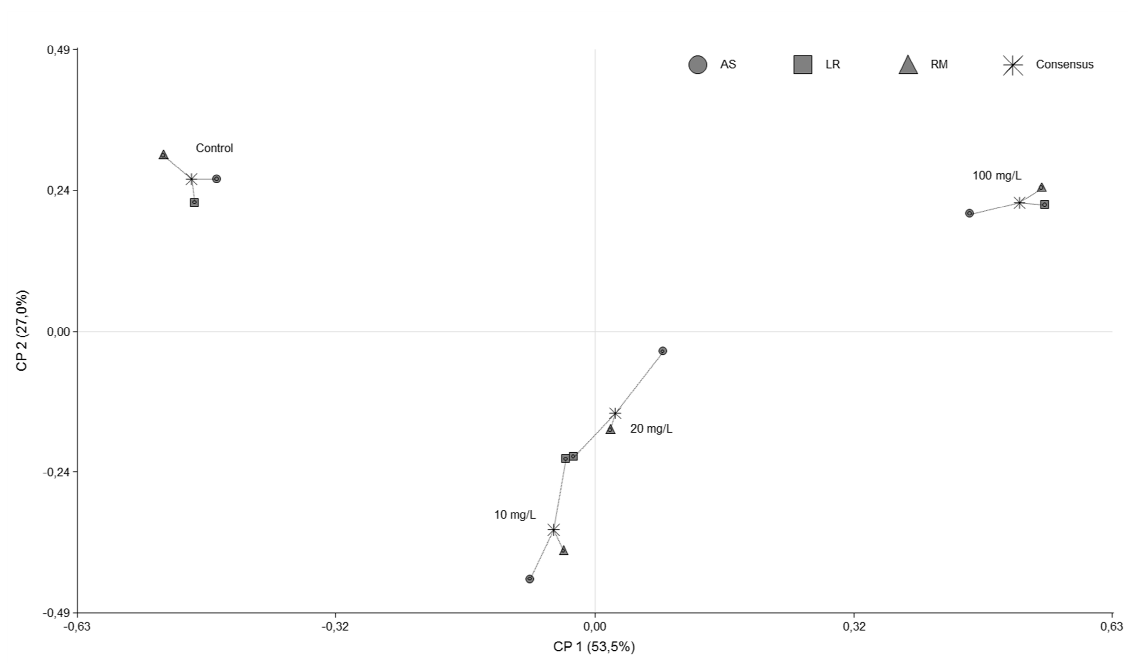


Figure 7



TABLES

Table 1

Iron concentration ($\mu\text{g g}^{-1}$ dry weight) in different compartments of *R. mangle*: Fe-plaque (external iron; calculated from DCB extract, measured by ICP-MS), roots (internal iron; acid digestion of roots after removing Fe-plaque by DCB method), and total iron (Fe-plaque + roots). Percentage of extracted iron was calculated from the ratio between Fe-plaque and total iron. Reported values correspond to mean $\pm$ SD. Significant differences between three DCB protocols are indicated by different letters in the same row (Tukey test, $P < 0.05$).

Plant compartment	DCB extraction protocols for Fe-plaque as reported in the literature		
	Taylor and Crowder (1983)	Otte et al. (1991)	Liu et al. (2004)
[Fe] plaque	0.38 ± 0.15 a	0.16 ± 0.03 b	0.28 ± 0.07 ab
[Fe] root	0.25 ± 0.05 b	0.73 ± 0.20 ab	0.46 ± 0.25 a
[Fe] total	0.63 ± 0.20 a	0.90 ± 0.22 a	0.73 ± 0.29 a
% extracted Fe	0.60 ± 0.04 a	0.19 ± 0.04 c	0.40 ± 0.11 b

Table 2

Variations (compared to controls) in epidermis thickness, cortex thickness, vascular cylinder diameter, root diameter and air gap area in roots of *A. schaueriana*, *L. racemosa* and *R. mangle*, exposed to different amounts of added-Fe(II). Values are reported as mean $\pm$ SD. Significant differences for the same parameter, analyzed between different exposure concentrations for each plant, are pointed out using lowercase letters. Significant differences between three studied species, with the same exposure concentration, are reported with uppercase letters. Different letters correspond to significant differences (Tukey test, $P < 0.05$).

		<i>A. schaueriana</i>	<i>L. racemosa</i>	<i>R. mangle</i>	
Fe concentrations	10 mg L ⁻¹	Epidermis	0.80 ± 0.09 aB	1.00 ± 0.09 aAB	1.17 ± 0.42 aA
		Cortex	1.25 ± 0.32 aA	0.70 ± 0.15 aA	1.16 ± 0.52 aA
		Vascular cylinder	1.32 ± 0.32 aA	0.97 ± 0.15 aA	1.12 ± 0.56 aA
		Root	1.32 ± 0.33 aA	0.90 ± 0.09 abA	1.16 ± 0.51 aA
		Air gap	1.45 ± 0.78 aA	1.85 ± 2.04 aA	1.03 ± 0.67 aA
	20 mg L ⁻¹	Epidermis	0.67 ± 0.11 aB	1.10 ± 0.23 aA	0.96± 0.20 aAB
		Cortex	0.67 ± 0.17 bB	0.97 ± 0.11 aA	1.15 ± 0.37 aA
		Vascular cylinder	1.13 ± 0.35 aA	1.20 ± 0.34 aA	1.01 ± 0.19 aA
		Root	0.81 ± 0.23 bA	1.05 ± 0.11 aA	1.10 ± 0.28 aA
		Air gap	0.78 ± 0.93 aB	2.94 ± 0.98 aA	0.94 ± 0.34 aB
	100 mg L ⁻¹	Epidermis	0.80 ± 0.05 aA	0.98 ± 0.30 aA	1.16 ± 0.30 aA
		Cortex	0.70 ± 0.11 bA	0.72 ± 0.31 aA	1.03 ± 0.41 aA
		Vascular cylinder	0.93 ± 0.21 aA	0.87 ± 0.12 aA	0.97 ± 0.31 aA
		Root	0.76 ± 0.13 bA	0.75 ± 0.18 bA	1.04 ± 0.39 aA
		Air gap	0.81 ± 0.59 aB	1.53 ± 0.36 aA	0.74 ± 0.44 aB

Supplementary material

Table 1S: Iron concentration ($\mu\text{g g}^{-1}$ dry weight) in Fe-plaque, roots, rhizophores, stems, leaves and salt glands secretion ($\mu\text{g g}^{-1}$ dry weight or $\mu\text{g cm}^{-2}$) of *A. schaueriana*, *L. racemosa* and *R. mangle*, including density of salt glands in leaves of *A. schaueriana* e *L. racemosa*. Reported values correspond to median $\pm$ first quartil. Significant differences for the same parameter, analyzed between different exposure concentrations for each plant, are pointed out using lowercase letters. Significant differences between three studied species, with the same exposure concentration, are reported with uppercase letters. Different letters correspond to significant differences (Kruskal-Wallis test, $P < 0.05$). TFsr: translocation factor between stems and roots; TFls: translocation factor between leaves and stems; TFlr: translocation factor between leaves and roots; TFsh: translocation factor between aerial parts (stems + leaves) and roots; Upper glands: salt glands in upper surface of leaves; Lower glands: salt glands in lower surface of leaves; NA: not available.

Fe concentrations		<i>A. schaueriana</i>	<i>L. racemosa</i>	<i>R. mangle</i>
	Plaque	134.25 $\pm$ 128.62 aA	464.58 $\pm$ 209.28 cA	32.72 $\pm$ 31.83 cB
	Root	28.86 $\pm$ 18.12 bAB	177.08 $\pm$ 150.46 cA	4.96 $\pm$ 1.35 cB
	Rhizophore	NA	NA	1.55 $\pm$ 1.38 a
	Stem	43.68 $\pm$ 37.11 abA	11.82 $\pm$ 4.11 bB	6.85 $\pm$ 4.37 aB
	Leaf	129.77 $\pm$ 94.04 aA	38.67 $\pm$ 24.24 aB	25.21 $\pm$ 23.05 aB
	Secretion (g)	8.36 $\pm$ 8.36 aB	33.81 $\pm$ 33.81 aA	NA
	Secretion (cm^2)	0.06 $\pm$ 0.06 aB	0.24 $\pm$ 0.19 aA	NA
	Tfsr	1.99 $\pm$ 1.72 aA	0.05 $\pm$ 0.01 aB	1.81 $\pm$ 1.13 aA
	Tfls	2.58 $\pm$ 2.26 aA	3.27 $\pm$ 2.36 aA	3.37 $\pm$ 3.26 aA
	Tflr	4.50 $\pm$ 3.19 aA	0.21 $\pm$ 0.18 aB	5.45 $\pm$ 3.93 aA
	Tfsh	6.48 $\pm$ 4.43 aA	0.23 $\pm$ 0.23 aB	8.68 $\pm$ 4.81 aA
	Upper glands	17.48 $\pm$ 16.94 aA	0.98 $\pm$ 0.81 aB	NA
	Lower glands	8.27 $\pm$ 7.86 aA	0.27 $\pm$ 0.14 aB	NA
	Plaque	89.14 $\pm$ 66.29 cAB	838.59 $\pm$ 838.59 bcA	35.87 $\pm$ 33.92 bcB
	Root	30.48 $\pm$ 20.74 bB	406.27 $\pm$ 392.55 bcA	32.16 $\pm$ 31.95 bcB
	Rhizophore	NA	NA	1.29 $\pm$ 1.27 a
	Stem	52.45 $\pm$ 35.92 abA	39.69 $\pm$ 3.33 bAB	11.36 $\pm$ 1.37 aB
	Leaf	104.03 $\pm$ 96.71 aA	57.67 $\pm$ 53.24 aAB	47.73 $\pm$ 46.89 aB
	Secretion (g)	3.69 $\pm$ 3.69 bA	0.04 $\pm$ 0.01 bA	NA
	Secretion (cm^2)	0.03 $\pm$ 0.02 bA	0.04 $\pm$ 0.03 bcA	NA
	Tfsr	1.77 $\pm$ 1.18 abA	0.09 $\pm$ 0.01 aB	0.29 $\pm$ 0.06 abB
	Tfls	2.69 $\pm$ 1.19 abA	1.68 $\pm$ 1.56 aA	4.40 $\pm$ 3.96 aA
	Tflr	3.17 $\pm$ 2.10 abA	0.14 $\pm$ 0.12 aB	1.44 $\pm$ 1.36 abAB
	Tfsh	4.35 $\pm$ 3.87 aA	0.22 $\pm$ 0.15 aB	1.74 $\pm$ 1.57 abAB
	Upper glands	25.07 $\pm$ 21.00 aA	1.22 $\pm$ 0.95 aB	NA
	Lower glands	9.62 $\pm$ 7.18 aA	0.27 $\pm$ 0.27 aB	NA

Table 1S (cont.)

	<i>A. schaueriana</i>	<i>L. racemosa</i>	<i>R. mangle</i>
Plaque	107.52 ± 107.25 bcAB	1302.34 ± 1302.34 abA	62.49 ± 51.81 abB
Root	35.49 ± 34.03 abB	451.85 ± 408.73 abA	75.38 ± 71.37 abAB
Rhizophore	NA	NA	1.42 ± 1.07 a
Stem	34.10 ± 16.47 bA	32.91 ± 18.22 bA	5.04 ± 1.44 aB
Leaf	95.52 ± 72.84 aA	77.88 ± 46.08 aAB	44.67 ± 25.72 aB
Secretion (g)	7.49 ± 5.62 aA	1.67 ± 0.05 bB	NA
Secretion (cm²)	0.05 ± 0.04 abB	0.23 ± 0.23 abA	NA
Tfsr	1.15 ± 0.46 bcA	0.06 ± 0.04 aB	0.07 ± 0.02 bcB
Tfls	2.80 ± 2.15 aA	2.01 ± 1.94 aA	9.70 ± 3.22 aA
Tflr	2.92 ± 2.14 abA	0.12 ± 0.10 abB	0.56 ± 0.38 bcAB
Tfsh	3.55 ± 3.29 abA	0.18 ± 0.13 aB	0.58 ± 0.40 bcAB
Upper glands	22.49 ± 20.46 aA	1.36 ± 1.08 aB	NA
Lower glands	8.40 ± 7.72 aA	0.54 ± 0.14 aB	NA
Plaque	125.71 ± 125.68 abB	2646.07 ± 2365.11 aA	334.98 ± 223.17 aAB
Root	57.98 ± 55.53 aB	601.86 ± 557.76 aA	230.59 ± 199.80 aAB
Rhizophore	NA	NA	1.57 ± 1.47 a
Stem	80.48 ± 1.16 aA	56.97 ± 50.23 aAB	1.42 ± 1.41 aB
Leaf	102.84 ± 92.27 aA	42.24 ± 9.56 aB	37.46 ± 28.51 aB
Secretion (g)	3.38 ± 3.38 bA	0.03 ± 0.0034 bB	NA
Secretion (cm²)	0.03 ± 0.03 b	0.0003 ± 0.00004 c	NA
Tfsr	0.02 ± 0.02 cA	0.10 ± 0.08 aA	0.01 ± 0.005 cB
Tfls	1.37 ± 1.45 bB	0.68 ± 0.17 bB	29.29 ± 19.53 aA
Tflr	1.77 ± 1.60 bA	0.07 ± 0.02 bB	0.17 ± 0.06 cB
Tfsh	1.79 ± 1.61 bA	0.17 ± 0.14 aB	0.18 ± 0.07 cB
Upper glands	16.40 ± 14.36 aA	1.76 ± 0.63 aB	NA
Lower glands	8.27 ± 8.13 aA	0.41 ± 0.41 aB	NA

Table 2S: Iron concentration ($\mu\text{g g}^{-1}$ dry weight) in Fe-plaque, roots, rhizophores, stems, leaves and salt glands secretion of *A. schaueriana*, *L. racemosa* and *R. mangle*. Reported values correspond to median $\pm$ first quartil. Significant differences between measured variables, corresponding to the same amount of added-iron, are indicated by different letters (Kruskal-Wallis test, $P < 0.05$). NA: not available.

		<i>A. schaueriana</i>	<i>L. racemosa</i>	<i>R. mangle</i>
Fe concentrations	0 mg L ⁻¹			
	Plaque	134.25 $\pm$ 128.62 a	464.58 $\pm$ 209.28 a	32.72 $\pm$ 31.83 a
	Root	28.86 $\pm$ 18.12 bc	177.08 $\pm$ 150.46 ab	4.96 $\pm$ 1.35 c
	Rhizophore	NA	NA	1.55 $\pm$ 1.38 c
	Stem	43.68 $\pm$ 37.11 ab	11.82 $\pm$ 4.11 cd	6.85 $\pm$ 4.37 bc
	Leaf	129.77 $\pm$ 94.04 a	38.67 $\pm$ 24.24 bc	25.21 $\pm$ 23.05 ab
	Secretion (g)	8.36 $\pm$ 8.36 bc	33.81 $\pm$ 33.81 bc	NA
	Secretion (cm ²)	0.06 $\pm$ 0.06 c	0.24 $\pm$ 0.19 d	NA
10 mg L ⁻¹	Plaque	89.14 $\pm$ 66.29 ab	838.59 $\pm$ 838.59 a	35.87 $\pm$ 33.92 ab
	Root	30.48 $\pm$ 20.74 bc	406.27 $\pm$ 392.55 a	32.16 $\pm$ 31.95 ab
	Rhizophore	NA	NA	1.29 $\pm$ 1.27 c
	Stem	52.45 $\pm$ 35.92 ab	39.69 $\pm$ 3.33 bc	11.36 $\pm$ 1.37 bc
	Leaf	104.03 $\pm$ 96.71 a	57.67 $\pm$ 53.24 ab	47.73 $\pm$ 46.89 a
	Secretion (g)	3.69 $\pm$ 3.69 cd	0.04 $\pm$ 0.01 c	NA
	Secretion (cm ²)	0.03 $\pm$ 0.02 d	0.04 $\pm$ 0.03 c	NA
20 mg L ⁻¹	Plaque	107.52 $\pm$ 107.25 a	1302.34 $\pm$ 1302.34 a	62.49 $\pm$ 51.81 a
	Root	35.49 $\pm$ 34.03 abc	451.85 $\pm$ 408.73 ab	75.38 $\pm$ 71.37 a
	Rhizophore	NA	NA	1.42 $\pm$ 1.07 b
	Stem	34.10 $\pm$ 16.47 bc	32.91 $\pm$ 18.22 bc	5.04 $\pm$ 1.44 b
	Leaf	95.52 $\pm$ 72.84 ab	77.88 $\pm$ 46.08 ab	44.67 $\pm$ 25.72 ab
	Secretion (g)	7.49 $\pm$ 5.62 cd	1.67 $\pm$ 0.05 c	NA
	Secretion (cm ²)	0.05 $\pm$ 0.04 d	0.23 $\pm$ 0.23 c	NA
100 mg L ⁻¹	Plaque	125.71 $\pm$ 125.68 a	2646.07 $\pm$ 2365.11 a	334.98 $\pm$ 223.17 a
	Root	57.98 $\pm$ 55.53 bc	601.86 $\pm$ 557.76 ab	230.59 $\pm$ 199.80 a
	Rhizophore	NA	NA	1.57 $\pm$ 1.47 b
	Stem	80.48 $\pm$ 1.16 ab	56.97 $\pm$ 50.23 bc	1.42 $\pm$ 1.41 b
	Leaf	102.84 $\pm$ 92.27 ab	42.24 $\pm$ 9.56 bc	37.46 $\pm$ 28.51 ab
	Secretion (g)	3.38 $\pm$ 3.38 c	0.03 $\pm$ 0.0034 cd	NA
	Secretion (cm ²)	0.03 $\pm$ 0.03 c	0.0003 $\pm$ 0.00004 d	NA

Artigo 2

Iron elimination through salt glands in leaves of two mangrove species

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Abstract

Salt glands are specialized epidermal cells present in leaves of halophytes species capable of controlling the internal balance of salt. However, some studies have found that these structures can eliminate metals unrelated to salt balance. During a histochemical study, using a specific marker for iron, we observed the presence of iron in salt glands of *Avicennia schaueriana* and *Laguncularia racemosa*, which is not yet reported in the literature. Thus, this study aimed at investigating the iron elimination by salt glands present in leaves of these species. Salt glands from both species showed positive histochemical reaction when tested with Perl's reagent, indicating iron accumulation. Additionally, ultracitochemistry, using the same specific marker, showed that iron was in secretory cells, adhered to the membrane of vacuoles and to plasmodesmata. In both species, the presence of iron in the exudate was confirmed by GF-AAS ($9.4 \text{ ng cm}^{-2} \text{ d}^{-1}$ and $1025 \text{ ng g}^{-1} \text{ d}^{-1}$ in *A. schaueriana*; $11.6 \text{ ng cm}^{-2} \text{ d}^{-1}$ and $1039 \text{ ng g}^{-1} \text{ d}^{-1}$ in *L. racemosa*). To our knowledge, this is the first report on iron elimination by salt glands in leaves of mangrove plants, which could explain the higher iron content found in leaves of species containing salt glands (*A. schaueriana* and *L. racemosa*) compared to species that do not have these structures (such as *Rhizophora mangle*).

Keywords: ultracitochemistry, iron, salt glands, mangrove plant, ultrastructure.

1. Introduction

Salt glands are specialized epidermal cells or trichomes, which play an active role in the elimination of dissolved mineral salts and sometimes organic substances. Salt glands vary in structure and in methods of salt release. In the secretory process, salt may be eliminated into vacuoles of the bladder cells, or to the outside of the glands through pores (Evert 2006). Salt glands occur in several families of eudicotyledons and one family of monocotyledons, Gramineae (Fahn 1979, 1988; Evert 2006). In eudicotyledons they are characteristic of the Frankeniaceae, Plumbaginaceae and Tamaricaceae, but also occur in some genera of other families, such as Chenopodiaceae, Convolvulaceae and Primulaceae. Salt glands are present in many mangrove plants, e.g. *Aegialitis* (Plumbaginaceae), *Aegiceras* (Myrsinaceae), *Avicennia* (Acanthaceae) and *Laguncularia* (Combretaceae) (Fahn 1988).

It is accepted that the salt glands occurring in the leaves of some mangrove species are adaptive structures to the high salt concentration in the sediment (Ball 1988; Sobrado 2004; Ye et al. 2005). These structures secrete ions, mainly Na^+ and Cl^- controlling the internal salt balance (Tomlinson 1994). In this process, other ions are also eliminated, such as K^+ , Mg^{2+} , Ca^{2+} , SO_4^{2-} , NO_3^- , PO_4^{3-} and HCO_3^- (Fahn 1988; Evert 2006). More recent studies have shown that other metals, such as Zn and Cu, can be secreted by salt glands. Furthermore, the metal secretion is increased as its concentration in the sediment rose, as it is the case with *Avicennia marina* (MacFarlane and Burchett 1999, 2000; Naidoo et al. 2014).

The mangrove swamp is a coastal ecosystem, located in the transition between marine and terrestrial ecosystems, which is subject to tidal regime (Tomlinson 1994). In general, mangrove sediments are hypoxic, rich in organic matter, having a high content of salt and metals (Ovalle et al. 1990). Among metals, iron presents high concentration in mangrove sediments (Defew et al. 2005).

Avicennia schaueriana and *Laguncularia racemosa* are two mangrove plant species, commonly found in the State of Espírito Santo (Brazil), affected by iron pollution arising from mine, industrial and harbor activities (Arrivabene et al. 2015). Both species have salt-secreting glands in their leaves (Tomlinson 1994) and are classified as salt-secreting (Parida and Jha 2010). During a field study, dedicated to verify the negative effects of iron on mangrove plants (Arrivabene et al. 2015), we observed the presence of iron within salt glands, a fact not yet reported in the literature. Thus, this study aimed at investigating the iron elimination by salt glands present in leaves of these species. We theorized that iron is being secreted through salt glands in these mangrove plants, probably contributing to the high iron-content in leaves of these two species exposed to environmental pollution with iron.

2. Materials and Methods

2.1. Study area

The field sampling was carried out in February 2010 (for chemical, anatomical and histochemical analysis), and in February 2015 (for ultracytochemical analysis). Sampling was performed during the low tide in the morning. The mangrove swamp area selected for sampling is located in the Ecological Mangrove Reserve of Rivers Piraquê-açu and Piraquê-Mirim, in the municipality of Aracruz, State of Espírito Santo (Brazil) (19°56'26"S and 40°13'27"W; Figure 1). This mangrove swamp covers 12.3 km² and it was selected because it is a well-preserved area (Jesus et al. 2009). The floristic composition of the grove consists of three tree species, *A. schaueriana*, *L. racemosa* and *R. mangle*. All samples were collected from individuals located at the edge of the mangrove. Exsiccates of studied species were deposited at the VIES herbarium at the Federal University of Espírito Santo (Brazil), identified with numbers 19649, 19650, and 19651. According to Kottek et al. (2006) the

climate classification in the State of Espírito Santo is equatorial (A); and in Aracruz the climate is equatorial savannah with a dry winter – Aw (≤ 60 mm rainfall during the winter).

2.2. Chemical analysis of iron

All glassware, plastic bottles/containers and ICP–MS PTFE probes and pipes were previously decontaminated using a neutral detergent Extran MA 02 (5% v/v), 15-50% v/v nitric acid (63.7%) sub-boiling grade and ultrapure water ($<5 \mu\text{g L}^{-1}$ TOC). Ultra-pure water was obtained from a purification system Arium 61316-RO, plus Arium 611 UV (Sartorius, Germany). Nitric acid sub-boiling grade was prepared from analytical grade acid, using a sub-boiling distiller (Figmay, Córdoba, Argentina). The purity of the nitric acid was checked by Mass Spectrometry Inductively Coupled Plasma (ICP-MS) (Agilent 7500cx, USA).

2.2.1. Iron in sediment

The analysis was carried out based on three independent sediment samples collected near to the rhizosphere of the studied species (0-20 cm depth). Samples were chilled, placed in pre-cleaned glass containers, and dried in a stainless steel oven (60°C) until constant weight. The dried material was crushed and sieved through a nylon mesh ($63 \mu\text{m}$) with an acrylic frame (to avoid contamination from metallic mesh). Dried sediment was digested using a mixture of nitric and hydrochloric acids in quartz close-vessels using a microwave oven (Anton Paar Multiwave 3000, Graz, Austria). Controls were prepared using the same protocol without sample (only reagents). The iron concentration was analyzed in triplicate using an ICP-MS (Agilent 7500cx, USA), in according to the method described by USEPA (2009). Quality assurance (QA) and quality control (QC) were performed using a certified reference material (CRM): EnviroMAT Sewage Sludge BE-1 as well as spiked samples

(doubling the starting iron concentration). CRM showed amounts within $\pm 5\%$ of the certified value. Spiked samples showed recoveries above 80%, as recommended by USEPA.

For the purpose of general characterization of the sediment, the analysis of organic matter, granulometry, pH and salinity was also carried out. The salinity was measured through the electrical conductivity (EC) of the interstitial water (bioavailable fraction), using an EC electrode Tetracon 325 (WTW, Multiline P4, Germany). The determination of pH, organic matter and granulometry were performed at the Agricultural Analysis and Consultancy Lab – Fullin, Linhares, Espírito Santo. The pH was determined using a pH meter DIGIMED model DM 22 (São Paulo, Brazil). Organic matter (OM) content was extracted by oxidation with $\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ (4 mol L^{-1}) + H_2SO_4 (10 mol L^{-1}), and measured by atomic absorption spectrometry (Buck Scientific, Model 210 VGP, East Norwalk, USA) according to Raji et al. (2001). Sediment granulometry was determined by densitometry (Embrapa 1997), and classified according to the Brazilian Society of Soil Science.

2.2.2. Iron in leaves

Leaves collected from four individuals per species were used. All samples were washed with RO-water, dried at 37°C until constant weight, grinded and homogenized with mortar and pestle. Samples were then digested using nitric acid and hydrochloric acid (37%, ultra-pure, sub-boiling grade) ($\text{HNO}_3\text{:HCl} = 4\text{:}1$), in pre-cleaned PTFE tubes (Sevillanex, USA) for 24 h. The digested material was filtered ($0.45 \mu\text{m}$, Millipore, USA) and stored at 4°C until analysis. Controls were prepared using the same protocol without sample (only reagents).

The iron concentration was analyzed in triplicate using an ICP–MS (Agilent 7500cx, USA), following the method described by USEPA (2009). Quality assurance (QA) and quality control (QC) were done using the CRM peach leaves NIST SRM 1547. CRM showed

amounts within $\pm 5\%$ of the certified value. Spiked samples were also prepared to verify the matrix effect. For this, standard solutions Merck VI CertiPUR[®] (Merck Chemicals, Buenos Aires, Argentina) were added to a known amount of dried samples; the rest of the procedure was the same used for non-spiked samples. After analysis, spiked samples showed an average recovery of $95 \pm 13\%$.

To determine the degree of iron accumulation in each plant species, the bio-concentration factor (BCF) was calculated as the ratio of the iron concentration in leaves to the iron concentration in the corresponding sediment.

2.2.3. Iron in the secretion from salt glands

Leaves from five individuals of each species were washed using a neutral detergent Extran MA 02 (González and González-Chávez 2006), and then rinsed several times with RO-water. The sampled branches were covered with plastic bags to prevent deposits of particulate matter or to prevent washing by rainfall. We were careful to use transparent plastic bags in order to minimize interference in the photosynthetically active radiation on the leaves, which could cause stomatal closure and consequent reduction of transpiration flow and secretion as observed by Scholander et al. (1962). Moreover, small openings were made at the bottom of the bags to avoid the saturation of the air with the resulting humidity of this closed system, which could also cause a decrease in the transpiration flow. After 24 h, the leaves were immersed for about 3 seconds in TPP tubes, containing 50 mL of 0.1% v/v ultrapure nitric acid (adapted from Manousaki et al. 2008). Each analysis was performed in triplicate, using a graphite furnace atomic absorption spectrometer (GF-AAS) (ZEEnit 700, Analytik Jena, Germany), equipped with an autosampler (MPE 60, Jena, Germany). Due to the lack of certified reference material, both an analyte addition plot and recovery tests were used to ensure result reliability (QA + QC). Recoveries ranged between 98 and 103%.

In order to calculate the secretion rate per area and mass unit, both leaf area (cm²) and leaf dry mass (g) of the leaves used to collect the secretion were measured. Leaf area was measured with the Area Meter LI-COR 3100 (Lincoln, USA), while the dry mass was obtained by weighing the samples after they were oven-dried at 60°C until constant weight.

2.3. Leaf anatomical analysis

Leaves of four randomly sampled independent individuals were collected to study leaf anatomy. Samples of the middle third of the leaf blade were fixed in FAA 50 (Johansen 1940), and stored in 70% ethanol. The material was dehydrated in a graded ethylic series, embedded in methacrylate historesin (Leica®) according to the manufacturer instructions. Cross sections (8 µm in thickness) were obtained using a rotary microtome. The cuts were dyed with 0.05% toluidine blue (O'Brien et al. 1964) and mounted in Canada balsam. The sections were documented using an image capture system coupled to a Nikon E200 microscope (Tokyo, Japan).

2.4. Iron histochemistry staining of leaves

Samples of fully expanded leaves from four independent individuals of *L. racemosa* and *A. schaueriana*, located on the third node from the apical bud, were collected and rinsed with RO-water to remove any particulate deposits on the leaf surface. To detect Fe(III) in leaf tissues, cross sections of fresh plant material, hand-free gathered, were immersed in Perl's reagent (Bancroft et al. 2008). After 24 h of reaction, these sections were rinsed and mounted in RO-water between a slide and a coverslip. The reaction was considered positive for Fe(III) in areas showing Prussian blue color. Negative controls, consisting of samples without Perl's reagent, were also analyzed. Sections were documented using a photomicroscope Nikon Eclipse 50i (Tokyo, Japan).

2.5. Ultracytochemical detection of iron in salt glands

Leaf samples located on the first node from the apical bud were collected and fixed using 3% glutaraldehyde solution in 0.1M cacodylate buffer (pH 7.3). After 24 h, samples were immersed in Perl's reagent to evidence the presence of Fe(III) as previously described. Samples were rinsed using a sucrose-cacodylate buffer, post-fixed for 1 h in 1% OsO₄ in 0.1 M cacodylate buffer, dehydrated in alcohol series and propylene oxide, soaked in low-viscosity medium Spurr (adapted from Parmley et al. 1978). Then, ultrafine sections (80 nm) were contrasted using uranyl acetate and lead citrate (Reynolds 1963), followed by analysis in a transmission electron microscope Tecnai Spirit (Fei Co., Hillsboro, USA). Sections without contrast were analyzed in parallel for better identification of the reaction, which is observed by stain deposits, generally cuboidal or irregular in shape.

2.6. Test to verify the iron elimination

To verify that the iron would be leaving from the salt glands and not coming through external contamination, three propagules of each species were collected at the ecological reserve of the Lameirão county (Estação Ecológica Municipal Ilha do Lameirão), State of Espírito Santo (Brazil), during the spring 2013 (*A. schaueriana*) and the summer 2014 (*L. racemosa*). After sampling, propagules were transported to a greenhouse at the Federal University of Espírito Santo, where they were cultivated in PVC pots (2.8 L each) containing washed sand. Sand pots were stored in receptacles containing a nutritive medium (Hoagland and Arnon 1950), renewed weekly, with 0.25 ionic strength and a salt content of 7 g L⁻¹. Propagules were developed under these conditions during eight months. Then, for 1 month, plants were not fed with iron in nutrient solution and after this period, some leaves were sprayed for 3 days with a solution 1 µM of Fe as Fe₂SO₄. Leaves that had and had not been

sprayed with iron in solution were collected for histochemical detection of Fe as describe in the section 2.4.

3. Results

3.1. Physical and chemical analysis of sediment

The sediment of the sampled mangrove area in Aracruz is composed mostly of silt and clay; classified as silty clay. The organic matter content was $111 \pm 8 \text{ g dm}^{-3}$. The average salinity was $35 \pm 5 \text{ psu}$ and the pH of the area is slightly acidic (6.7 ± 0.5) (Table 1).

3.2. Iron concentration in sediment, leaves and secretion from salt glands

The iron concentration in sediment was $19.9 \pm 0,5 \text{ mg g}^{-1}$. Higher values of iron were observed in the leaf tissue of *A. schaueriana* ($0.28 \pm 0.02 \text{ mg g}^{-1}$) than in *L. racemosa* ($0.18 \pm 0.02 \text{ mg g}^{-1}$), with bioconcentration factors of 0.014 and 0.009, respectively. The analysis of salt secretion in both species indicated the presence of iron, finding slightly higher values in *L. racemosa* ($12 \pm 4 \text{ ng cm}^{-2}$ and $1039 \pm 319 \text{ ng g}^{-1}$) compared to *A. schaueriana* ($9 \pm 3 \text{ ng cm}^{-2}$ and $1025 \pm 241 \text{ ng g}^{-1}$) (Table 2).

3.3. Anatomical analysis and histochemical detection of iron in leaves

Leaves of *A. schaueriana* have uniseriate epidermis with two types of salt-secreting structures: long-stalked and short-stalked glands. The first type is composed of stalk cell and secretory head and occur in small depressions of the upper surface, while those located in the lower surface generally occur in the same level as the other epidermal cells. The second type is also distributed on both sides of the epidermis, being more frequent on the upper surface, and is situated in small depressions. It is formed by collecting cells, a flattened cell stalk and secretory cells coated with cuticle. The leaves are hypostomatic and have dorsiventral

mesophyll with water storage parenchyma underlying the upper surface of the epidermis (Fig. 2A).

In *A. schaueriana*, there was a positive reaction to the Perl's reagent test in cells of collenchyma and cortical parenchyma in the midrib region. In the internerval area, additionally, there was accumulation of iron in cells of water storage and spongy parenchyma (Fig. 2B) – including those defining the substomatal chamber (Fig. 2C), as well as in long-stalked (Fig. 2D) and in short-stalked salt glands (Fig. 2E).

Leaves of *L. racemosa* have uniseriate epidermis with salt glands, which are distributed on both sides of the leaves. The salt glands consist of a secretory multicellular head, located at the bottom of a flask-shaped cavity, and the cavity wall cells, which are immersed in the palisade parenchyma. It is also possible to differentiate two glandular regions: the chamber or crypt, which is located secretory head to the depth, and the antechamber. The multicellular secretory head consists of collecting cells located at the base, stalk cells of flattened shape and a head with secreting cells themselves. The leaves are amphistomatic and have isobilateral mesophyll with water storage parenchyma in the central region (Fig. 3A).

Cross sections of the lamina of *L. racemosa* showed positive reaction to histochemical test, mainly in conductor cells and parenchymatic cells of xylem (Fig. 3C and 3D), but also in the phloem and cortical parenchyma cells in the midrib region (Fig. 3C). In the internerval area, there was accumulation of iron in the xylem, in the secretory head of salt glands (Fig. 3B), as well as in the gland's exudate (Fig. 3E).

3.4. Ultracytochemical detection of iron in salt glands

In *A. schaueriana*, stain deposits of cubic, irregular or circular shape were observed in short-stalked salt glands, indicating positive reaction to Perl's reagent (Fig. 4A). Iron

accumulation was detected in cuticle layers and in the cuticle proper (Fig. 4B), in the periclinal wall of the secretory cell wall in contact with the cuticle (Fig. 4C), and in the cell wall of secretory cells and plasmodesmata of stalk cell (Fig. 4D). Only short-stalked glands were analyzed because there were not obtained images of long-stalked glands in transmission electron microscopy, probably due to disruption of bladder cell.

In *L. racemosa*, there was positive reaction to Perl's reagent in secretory cells of glandular head, where stain deposits were observed adhered to the membrane of vacuoles (Fig. 5A and C), to plasmodesmata (Fig. 5D), and scattered throughout the dense cytoplasm (Fig. 5B and D). Furthermore, iron accumulation was detected in cytoplasm of stalk cell (Fig. 5B).

3.5 Test to verify the elimination of iron through salt glands

Findings describing the presence of iron within salt glands can be questioned, mainly because the observed iron could be introduced into the salt glands for diffusion from the external surface. So far, it is necessary to demonstrate that the observed iron arise from the internal part of the plant (root uptake and translocation until reach leaves and salt glands). The histochemical test done in leaves sprayed with the iron solution showed that this metal remained in the outer surface of long-stalked salt glands of *A. schaueriana*, without evidence of this metal within the gland (Fig. 6A). Also for *L. racemosa* iron was mainly located in the antechamber, the outer portion of salt glands. In some sections we observed presence of iron in the chamber of salt glands, *e.g.* in the inner portion of glands. However, we did not observe iron in the glandular head or xylem cells (Fig. 6B).

Leaves not sprayed with iron did not show presence of this metal in salt glands.

4. Discussion

The elimination of iron through salt glands of wild plants of *A. schaueriana* and *L. racemosa* was confirmed by histochemical analysis, ultracytochemical assays and by GF-AAS of the secretion. This secretion occurred under environmental conditions typical of the mangrove ecosystem, since the sampled area was characterized by silt clay sediment, rich in organic matter and with slightly acidic pH. To our knowledge, our current results constitute the first report on the elimination of iron by salt glands in leaves of mangrove plants.

Additionally, the histochemical analysis performed on leaves sprayed with an aqueous iron solution, and the corresponding non-sprayed control, allowed verifying that the iron observed in salt glands is coming probably from root-uptake. Conversely, iron added on the leave surface was not able to penetrate the interior of *A. schaueriana* salt glands. For *L. racemosa*, species having salt glands with a suitable shape to the entrance of contaminants, iron was not detected in the secretory head and xylem cells, although this metal has been detected in the material contained within the gland chamber. This ensures us that, in addition to all the care taken in field to protect the leaves against possible air pollution by particulate iron, the iron accumulation in salt glands revealed in histochemical analysis reflects the iron being eliminated by these structures, and not being absorbed by possible external contamination.

We do not rule out the hypothesis that the stomata are also involved in the release of iron to the leaf surface. The iron accumulation in cells of spongy parenchyma defining the substomatal chamber, observed by histochemical test, suggests a probable route for iron mobilization to both the salt gland and the stomata. Other research groups have reported the excretion of the metals (Cr, Cu, Pb, Zn and Hg) in leaves of *Phragmites australis*, a species that does not have salt glands (Burke et al. 2000; Windham et al. 2001; Weis et al. 2002). This last findings could be related to water loss by transpiration (Burke et al. 2000) rather than metal secretion as it is in our case.

A greater accumulation of iron was observed in leaves of *A. schaueriana* when compared to *L. racemosa*. It seems that *A. schaueriana* has a preferential accumulation, not only for iron, but for several metals, as have been reported in previous studies (Souza et al. 2014a, 2015). However there was no difference in iron concentration secreted between the two species.

The higher iron content found in leaves of species with salt glands (as *A. schaueriana* and *L. racemosa*) compared to species that do not have these structures (such as *Rhizophora mangle*) (Bernini et al. 2006; Cuzzuol and Campos 2001, Souza et al. 2014a, 2014b, 2015) can be explained by the presence of these structures. Differences found in the bioaccumulation of metals could be attributed to the regulatory mechanisms of salt in roots (Lacerda et al. 1985), with a higher amount of salt being absorbed by salt-secreting plants (salt-including). In these cases, the salt ultra-filtration mechanism present in roots could be responsible for this difference.

Intracellularly, most of the iron was found adhered to the vacuolar membrane of salt glands. The role of vacuoles in iron storage has been clearly demonstrated in *Arabidopsis*. That organelle generally functions as a metal pool to avoid toxicity, with various influx and efflux transporters involved in iron transport between the vacuole and cytosol (Kobayashi and Nishizawa 2012, Thomine and Vert 2013). Iron was also located in both in cell walls as in plasmodesmata of salt glands, highlighting the apoplastic and symplastic transport of this metal (Kobayashi and Nishizawa 2012). In *Avicennia marina*, a mangrove plant, Zn (MacFarlane and Burchett 1999, 2000) and Cu (MacFarlane and Burchett 2000) was in higher concentration in cell walls than cytoplasmic contents of all cell types of leaf tissue suggesting a large proportion of zinc is transported through leaf tissue apoplastically and or chelation of zinc to cell wall matrices was occurring. Several genes have been identified in subcellular transport of iron in plants (Thomine and Vert 2013) and some studies have verified the

accumulation (Lavid et al. 2001) and elimination (Gupta et al. 2006) of this metal in secretory structures. However, to the best of our knowledge, we found no studies that have evaluated the subcellular accumulation and iron transport in salt glands.

The Perl's reagent is one of several methods currently used to detect iron in plants the light microscopy level (Samira et al. 2013). However, the use of that reagent as an iron marker in the ultrastructural studies has been limited to animal cells, mostly duodenal mucosal cell of rats (Parmley 1978). In our study, we found that this technique applied in transmission electron microscopy can be successfully used in plant material, being a suitable method for analyzing the iron distribution in the cell plant.

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References

- Arrivabene HP, Souza IC, C3 WLO, Conti MM, Wunderlin DA, Milanez CRD (2015) Effect of pollution by particulate iron on the morphoanatomy, histochemistry, and bioaccumulation of three mangrove plant species in Brazil. *Chemosphere* 127:27-34.
- Ball MC (1988) Ecophysiology of mangroves. *Trees* 2:129-142.
- Bancroft JD, Stevens A, Turner DR (2008) Theory and practice of histological techniques. Churchill Livingstone, New York.
- Bernini E, Silva MAB, Carmo TMS, Cuzzuol GRF (2006) Composi33o qu3mica do sedimento e de folhas das esp3cies do manguezal do estu3rio do Rio S3o Mateus, Esp3rito Santo, Brasil. *Revista Brasil Bot* 29:689-699.
- Burke DJ, Weis JS, Weis P (2000) Release of metals by the leaves of the salt marsh grasses *Spartina alterniflora* and *Phragmites australis*. *Estuar Coast Shelf S* 51:153-159.
- Cuzzuol GRF, Campos A (2001) Aspectos nutricionais na vegeta33o de manguezal do estu3rio do Rio Mucuri, Bahia, Brasil. *Revista Brasil Bot* 24:227-234.
- Defew LH, Mair JM, Guzman HM (2005) An assessment of metal contamination in mangrove sediments and leaves from Punta Mala Bay, Pacific Panama. *Mar Pollut Bull* 50:547-552.
- Embrapa (1997) Manual de m3todos de an3lise de solo. Embrapa - Centro Nacional de Pesquisa de Solo, Rio de Janeiro.
- Evert RF (2006) Esau's plant anatomy: meristems, cells, and tissues of the plant body: their structure, function, and development. John Wiley & Sons, New Jersey.
- Fahn A (1979) Secretory tissues in plants. Academic Press, London.
- Fahn A (1988) Secretory tissues in vascular plants. *New Phytol* 108:229-257.
- Gonz3lez RC, Gonz3lez-Ch3vez MCA (2006) Metal accumulation in wild plants surrounding mining wastes. *Environ Pollut* 144:84-92.

- Gupta M, Biswas TK, Saha S, Debnst P (2006) Therapeutic utilization of secretory products of some Indian medicinal plants: a review. *Indian J Trad Knowledge* 5:569-575.
- Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. *California Agricultural Experiment Station* 347:1-32.
- Jesus HC, Santana EJ, Belo MJ, Silva LB, Ceotto CS, Neves DMC, Castro LB (2009) Avaliação da poluição ambiental dos rios Piraquê-açu e Piraquê-mirim (Santa Cruz – ES) através da análise de águas, biomonitoros e sedimentos. Relatório técnico – projeto Aracruz Celulose, Processo UFES nº 034373/2007-80.
- Johansen DA (1940) *Plant microtechnique*. McGraw-Hill Book Company Inc, New York.
- Kobayashi T, Nishizawa NK (2012) Iron uptake, translocation, and regulation in higher plants. *Annu Rev Plant Biol* 63:131-152.
- Kottek M, Grieser J, Beck C, Rudolf B, Rubel F (2006) World map of the Köppen–Geiger climate classification updated. *Meteorol Z* 15:259-63.
- Lacerda LD, Rezende CE, José DV, Wasserman JC, Francisco MC (1985) Mineral concentrations in leaves of mangrove trees. *Biotropica* 17:21-27.
- Lavid N, Barkay Z, Tel-Or E (2001) Accumulation of heavy metals in epidermal glands of the waterlily (*Nymphaeaceae*). *Planta* 212:313-322.
- MacFarlane GR, Burchett MD (1999) Zinc distribution and excretion in the leaves of the grey mangrove, *Avicennia marina* (Forsk.) Vierh. *Environ Exp Bot* 41:167-175.
- MacFarlane GR, Burchett MD (2000) Cellular distribution of copper, lead and zinc in the grey mangrove, *Avicennia marina* (Forsk.) Vierh. *Aquat Bot* 68:45-59.
- Manousaki E, Kadukova J, Papadantonakis N, Kalogerakis N (2008) Phytoextraction and phytoexcretion of Cd by the leaves of *Tamarix smyrnensis* growing on contaminated non-saline and saline soils. *Environ Res* 106:326-332.

- Naidoo G, Hiralal T, Naidoo Y (2014) Ecophysiological responses of the mangrove *Avicennia marina* to trace metal contamination. *Flora* 209:63-72.
- O'Brien TP, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma* 59:368–73.
- Ovalle ARC, Rezende CE, Lacerda LD, Silva CAR (1990) Factors affecting the hydrochemistry of a mangrove tidal creek, Sepetiba bay, Brazil. *Estuar Coast Shelf S* 31:639-650.
- Parida AK, Jha B (2010) Salt tolerance mechanisms in mangroves: a review. *Trees* 24:199-217.
- Parmley RT, Barton JC, Conrad ME, Austin RL (1978) Ultrastructural cytochemistry of iron absorption. *Am J Pathol* 93:707-718.
- Raij BV, Andrade JC, Cantarella H, Quaggio JA (2001) Análise química para avaliação da fertilidade de solos tropicais. Instituto Agronômico de Campinas, Campinas.
- Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J Cell Biol* 17:208-212.
- Samira R, Stallmann A, Massenburg LN, Long TA (2013) Ironing out the issues: integrated approaches to understanding iron homeostasis in plants. *Plant Sci* 210:250-259.
- Scholander PF, Hammel HT, Hemmingsen E, Garey W (1962) Salt balance in mangroves. *Plant Physiol* 37:722-729.
- Sobrado MA (2004) Influence of external salinity on the osmolality of xylem sap, leaf tissue and leaf gland secretion of the mangrove *Laguncularia racemosa* (L.) Gaertn. *Trees* 18:422-427.
- Souza I, Bonomo MM, Morozesk M, Rocha LD, Duarte ID, Furlan LM, Arrivabene HP, Monferrán MV, Matsumoto ST, Milanez CRD, Wunderlin DA, Fernandes MN (2014a) Adaptive plasticity of *Laguncularia racemosa* in response to different

- environmental conditions: integrating chemical and biological data by chemometrics. *Ecotoxicology* 23:335-348.
- Souza IC, Morozesk M, Duarte ID, Bonomo MM, Rocha LD, Furlan LM, Arrivabene HP, Monferrán MV, Matsumoto ST, Milanez CRD, Wunderlin DA, Fernandes MN (2014b) Matching pollution with adaptive changes in mangrove plants by multivariate statistics. A case study, *Rhizophora mangle* from four neotropical mangroves in Brazil. *Chemosphere* 108:115-124.
- Souza IC, Rocha LD, Morozesk M, Bonomo MM, Arrivabene HP, Duarte ID, Furlan LM, Monferrán MV, Matsumoto ST, Milanez CRD, Wunderlin DA, Fernandes MN (2015) Changes in bioaccumulation and translocation patterns between root and leaves of *Avicennia schaueriana* as adaptive response to different levels of metals in mangrove system. *Mar Pollut Bull* 94:176-184.
- Thomine S, Vert G (2013) Iron transport in plants: better be safe than sorry. *Curr Opin Plant Biol* 16:322-327.
- Tomlinson PB (1994) The botany of mangroves. Cambridge University Press, Cambridge.
- Usepa (2009) Risk-Based concentration table. Philadelphia PA. United States Environmental Protection Agency, Washington.
- Weis P, Windham L, Burke DJ, Weis JS (2002) Release into the environment of metals by two vascular salt marsh plants. *Mar Environ Res* 54:325-329.
- Windham L, Weis JS, Weis P (2001) Patterns and processes of mercury release from leaves of two dominant salt marsh macrophytes, *Phragmites australis* and *Spartina alterniflora*. *Estuaries* 24:787-795.
- Ye Y, Tam NFY, Lu CY, Wong YS (2005) Effects of salinity on germination, seedling growth and physiology of three salt-secreting mangrove species. *Aquat Bot* 83:193-205.

FIGURE CAPTIONS

Fig. 1 Location of study area (coordinates 19°56'26"S, 40°13'27"W)

Fig. 2 Leaf blade cross sections of *Avicennia schaueriana*. **A** Section stained with toluidine blue, **B-E** Detection of iron by histochemical test with Perl's reagent in internodal area. **B** General appearance, **C** Detail of stomata showing the detection of iron in the cells surrounding the substomatal chambers, **D** Detail of the long-stalked salt gland showing the iron inside (arrow), **E** Detail of the short-stalked salt gland showing the iron inside (arrow). (Pp = palisade parenchyma; Sg = short-stalked salt gland; Sp = spongy parenchyma; St = stomata; Lg = long-stalked salt gland; Wp = water storage parenchyma). Bars = 50 µm (**A-B**) and 10 µm (**C-E**)

Fig. 3 Leaf blade cross sections of *Laguncularia racemosa*. **A** Section stained with toluidine blue, **B-E** Detection of iron by histochemical test with Perl's reagent. **B** General appearance of internodal area, **C-D** Midrib. **C** General appearance, **D** Detail of the xylem. **E** Internodal area showing detail of the salt gland with positive reaction to the test in secretion (Arrow = secretor head of the salt gland; Ac = antechamber; Ch = chamber; Cp = cortical parenchyma; Ph = phloem; Pp = palisade parenchyma; Sg = salt gland; St = stomata; Wp = water storage parenchyma; Xy = xylem). Bars = 100 µm (**C**), 50 µm (**A-B**), 20 µm (**E**) and 10 µm (**D**)

Fig. 4 Cytochemical detection of Fe in short-stalked salt glands of *Avicennia schaueriana* observed under transmission electron microscope. Stain deposits indicate positive reaction to Perl's reagent. **A** General appearance of salt gland. **B-D** Details of the picture A. **B** Stain deposits scattered on the cuticle, with visible accumulation of Fe at the boundary between this

layer and the cuticle itself. **C** Accumulation of stain deposits on the outside of the external wall periclinal in contact with the cuticle. **D** Positive staining to Fe in the internal periclinal wall of secretory cell and plasmodesmata of stalk cell. (large arrow = stain deposits, thin arrow = plasmodesmata, * = space in cuticle, Cw = cell wall, Sc = secretory cell, St = stalk cell). Bars: 2 μm (A), 200 nm (B), 500 nm (C and D)

Fig. 5 Cytochemical detection of Fe in salt glands of *Laguncularia racemosa* observed under transmission electron microscope. Stain deposits indicate positive reaction to Perl's reagent. **A** General appearance of salt gland. **B-D** Details of the picture A. **B** Stain deposits scattered in the cytoplasm of the stalk cell and secretory cells. **C** Positive staining to Fe in the membrane of the vacuole of secretory cells. **D** Positive staining to Fe in cytoplasm and plasmodesmata. (large arrow = stain deposits, thin arrow = plasmodesmata, Cw = cell wall, Sc = secretory cell, St = stalk cell, Va = vacuole). Bars: 2 μm (A), 5 μm (B), 500 nm (C and D)

Fig. 6 Detection of iron by histochemical test with Perl's reagent in leaves sprayed with the iron solution. Leaf blade cross section of *Avicennia schaueriana* (**A**) and *Laguncularia racemosa* (**B**). Bars = 50 μm (A and B)

FIGURES

Figure 1

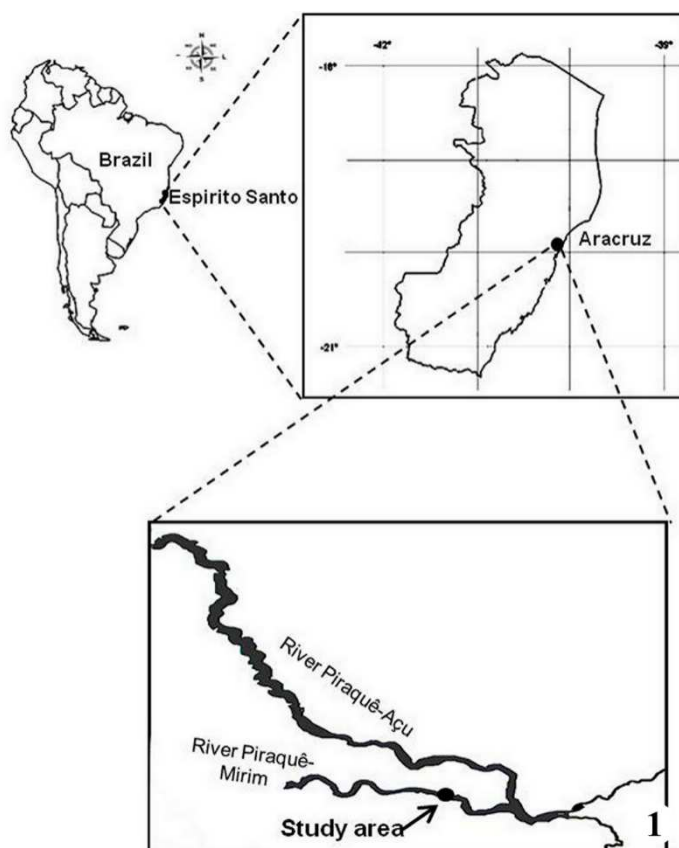


Figure 2

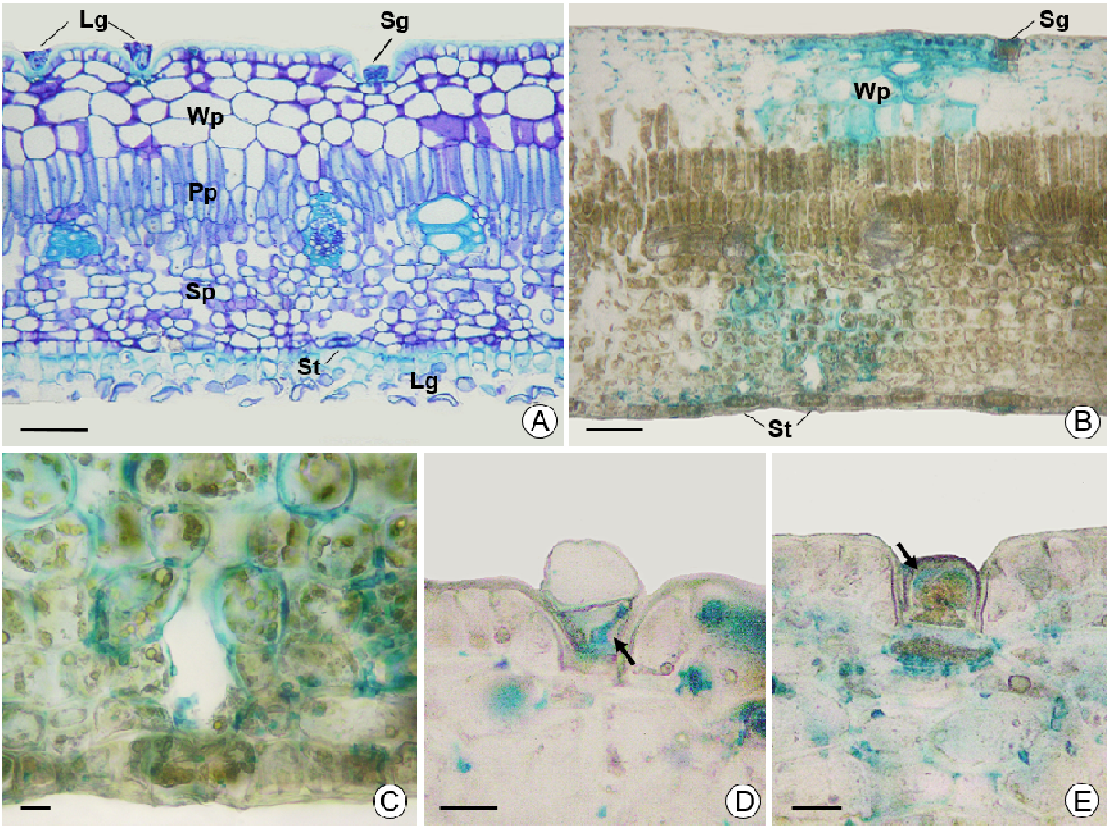


Figure 3

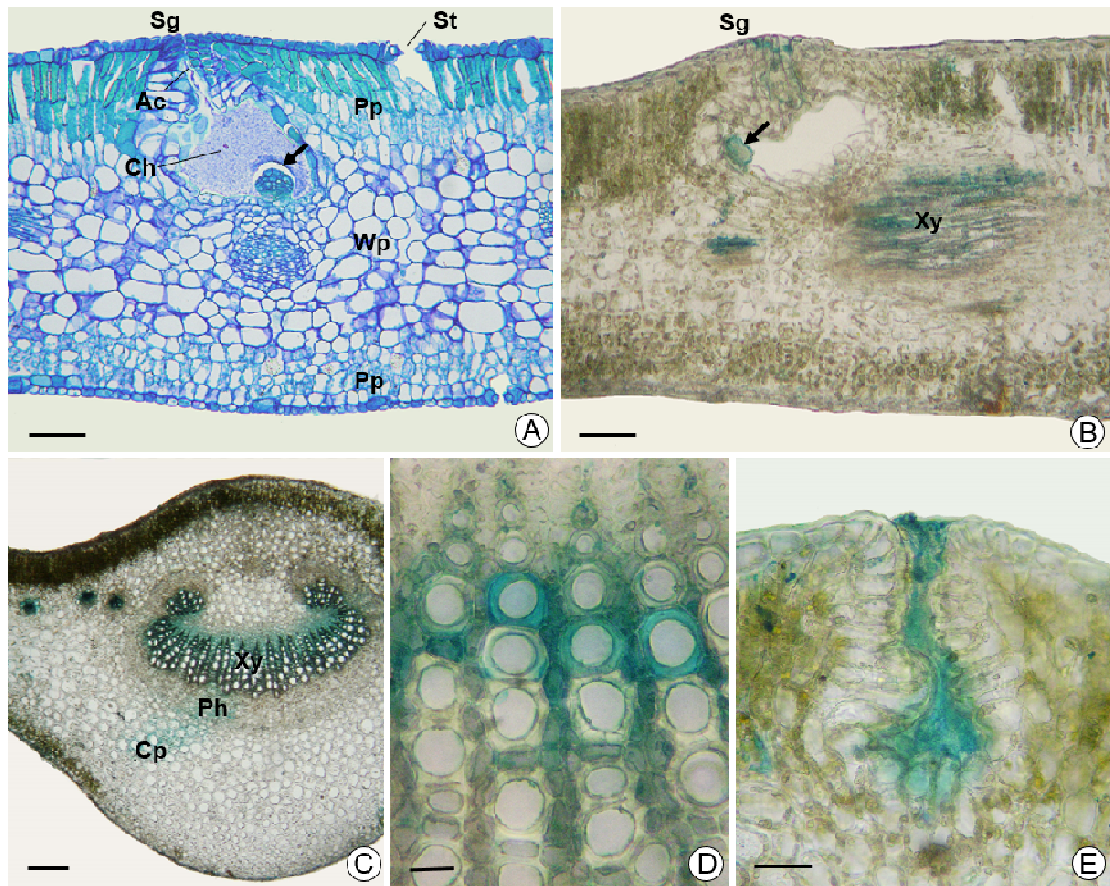


Figure 4

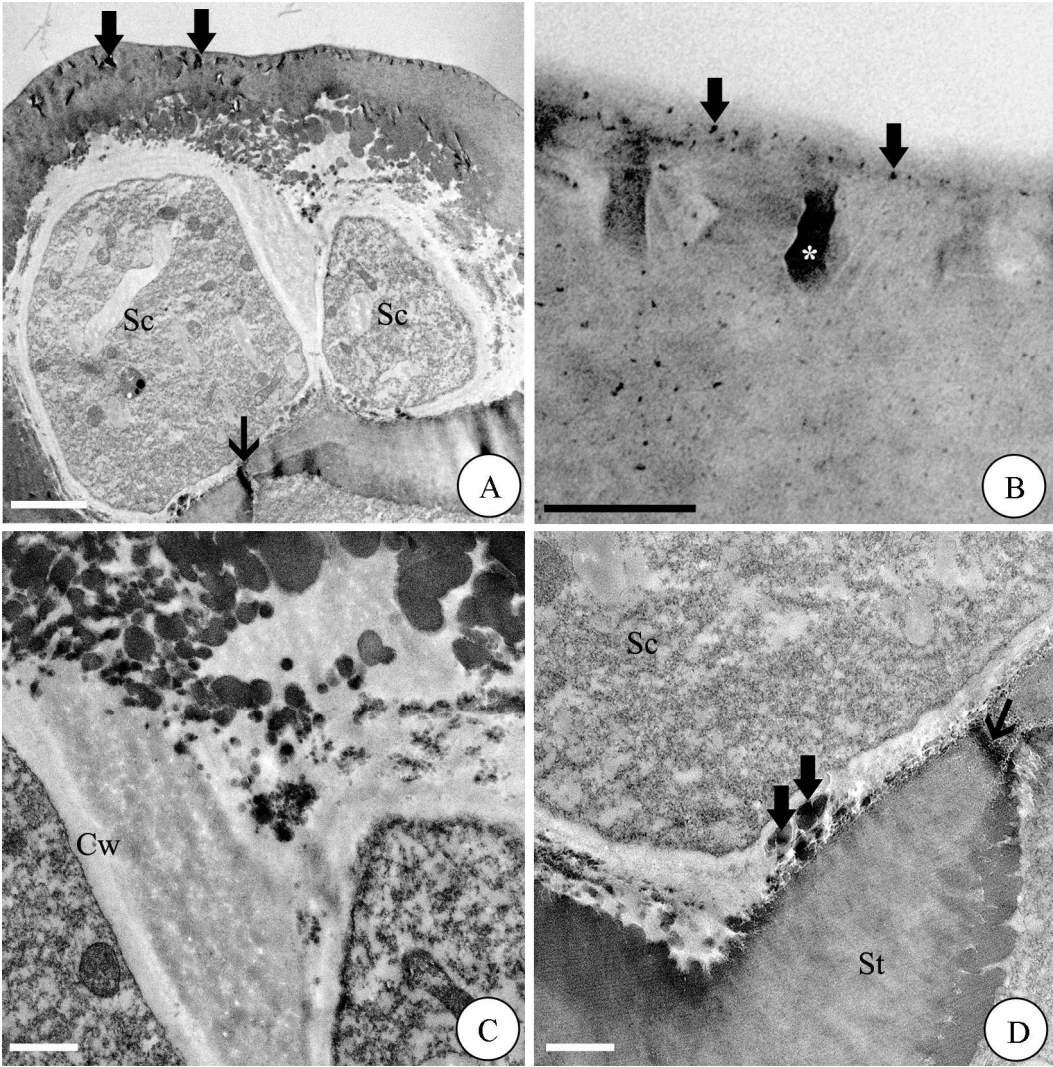


Figure 5

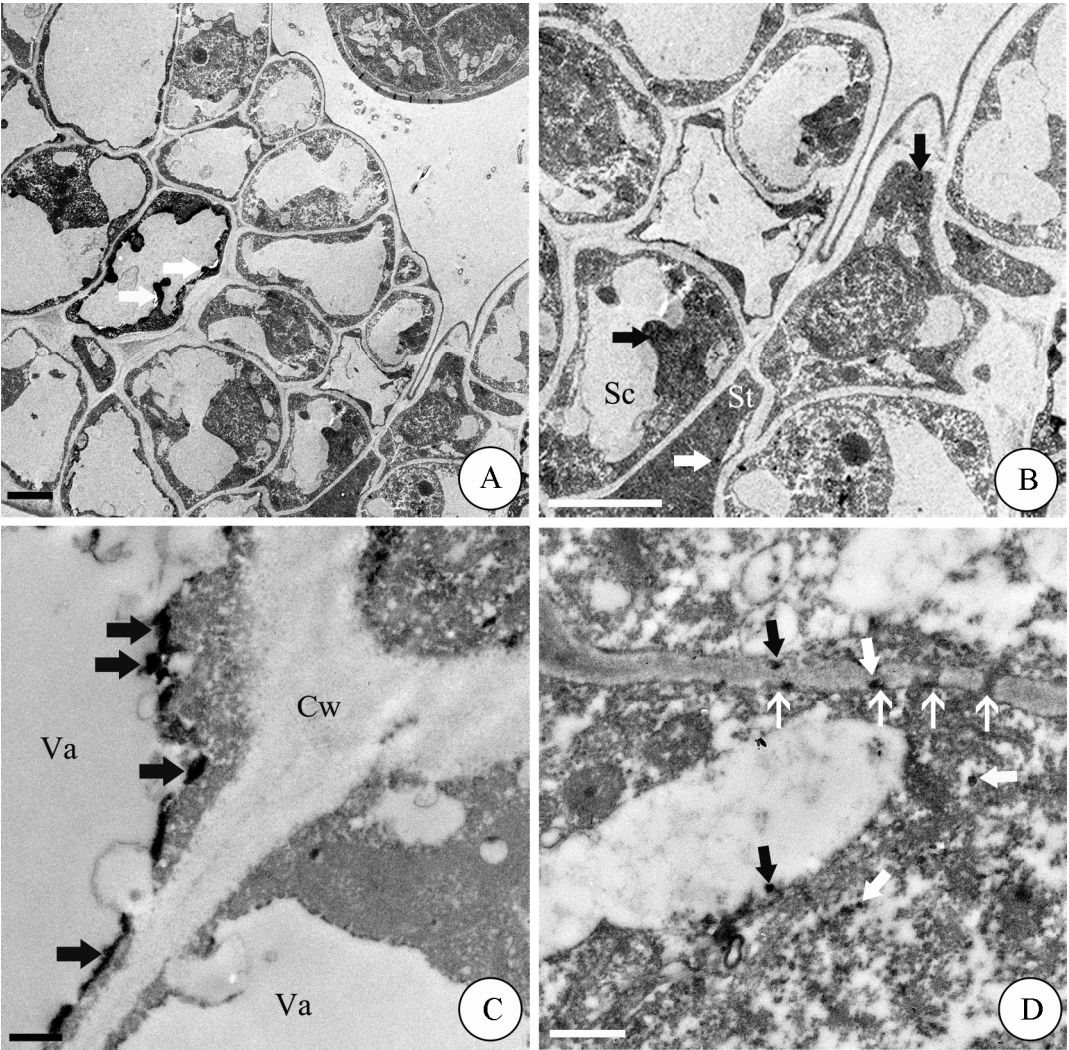
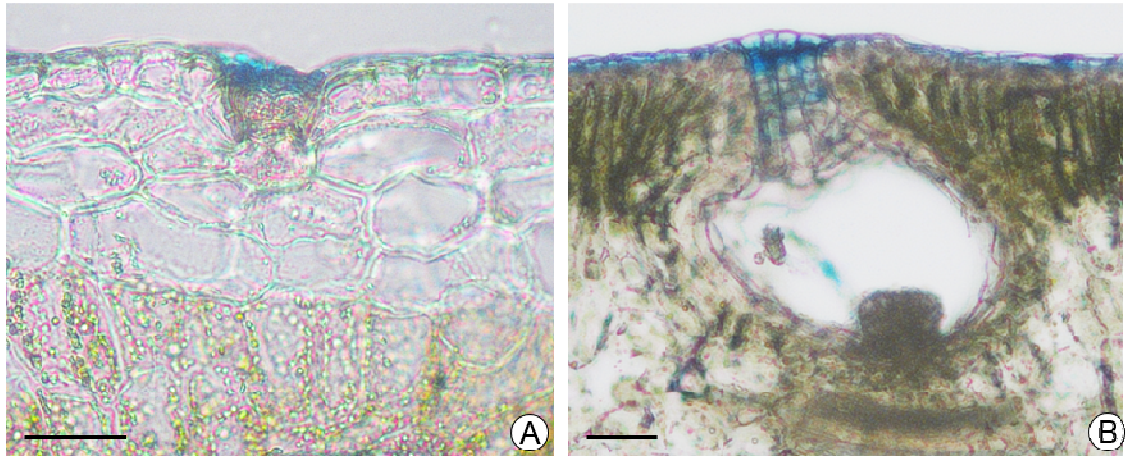


Figure 6



TABLES

Table 1. Granulometric fractions, textural classification, organic matter content (OM), pH and salinity of the mangrove sediment in Aracruz, Brazil. Values correspond to the mean $\pm$ standard deviation.

Granulometric fractions (%)				Textural classification	OM (g dm ⁻³)	pH	Salinity
Coarse sand	Fine sand	Silty	Clay				
5.5 $\pm$ 2.2	1.7 $\pm$ 0.5	51.4 $\pm$ 6.0	41.3 $\pm$ 2.3	Silty clay	111 $\pm$ 8	6.7 $\pm$ 0.4	34.7 $\pm$ 4.8

Table 2. Iron concentrations in sediment, leaves, salt secretion from salt glands and bioconcentration factors (CF) in *A. schaueriana* and *L. racemosa*, occurring in mangrove swamp in Aracruz, Brazil. Values correspond to the mean $\pm$ standard deviation.

	Sediment (mg g⁻¹)	Leaf (mg g⁻¹)	Secretion (ng g⁻¹)	Secretion (ng cm⁻²)	CF
<i>A. schaueriana</i>	19.9 $\pm$ 0.5	0.28 $\pm$ 0.02	1025 $\pm$ 241	9 $\pm$ 3	0.014
<i>L. racemosa</i>		0.18 $\pm$ 0.02	1039 $\pm$ 319	12 $\pm$ 4	0.009

Considerações Finais

Os resultados mostram que as plantas têm padrões diferenciais em relação à bioacumulação, translocação e secreção de ferro através de glândulas de sal.

Em geral, o aumento do teor de ferro no substrato acarretou em maior acúmulo desse elemento em placa de ferro, raízes e caules, mas não em folhas. Os menores fatores de translocação encontrados sob maiores doses de ferro mostram que o aumento do teor de ferro no substrato afetou significativamente a translocação desse elemento nas três espécies estudadas.

Não observamos maior teor de ferro em raiz quando comparado a parte aérea para *A. schaueriana*, diferente do comumente relatado em literatura. Assim, o maior teor de ferro acumulado em raízes mostrado em alguns estudos, pode ser devido à retenção desse metal na placa de ferro e não ao acúmulo real desse elemento nos tecidos radiculares.

Houve maior acúmulo de ferro em *L. racemosa* (para raiz) e *A. schaueriana* (para caule e folha) em relação a *R. mangle*, com menor acúmulo, o que destaca a absorção diferencial de elementos devido aos mecanismos reguladores de sal nas raízes.

Poucas alterações anatômicas foram encontradas em raízes sob diferentes doses de ferro. Não houve aumento da lacuna de ar e essa não se correlacionou com a placa de ferro, tão pouco com a quantidade de ferro acumulada nos tecidos vegetais.

A epiderme se confirmou como o principal local de acúmulo de ferro em raízes e, provavelmente, confere a principal barreira à entrada desse metal nas plantas. Contudo, histoquimicamente, não detectamos acúmulo de ferro na endoderme radicular.

A eliminação de ferro por glândulas de sal de *A. schaueriana* e *L. racemosa* foi indicada por meio de teste histoquímico e ultracitoquímico, e confirmada por meio de análise química. Contudo, diferente do relatado em literatura, não verificamos maior secreção de ferro sob concentrações crescentes desse elemento no substrato.

Para fins de monitoramento ambiental da poluição por ferro, avaliando-se as três espécies, *L. racemosa* foi a que apresentou melhor capacidade de uso como bioindicadora, uma vez que a bioacumulação de ferro em placa de ferro e raízes foi maior e aumentou progressivamente com o aumento de ferro no substrato. Além disso, nessa espécie, a quantidade de ferro secretada por glândulas de sal foi fortemente inibida quando os indivíduos foram expostos a maiores dosagens de ferro.

Ao nosso conhecimento, este é o primeiro estudo detalhado sobre a resposta de plantas de manguezal a concentrações crescentes de ferro sob condições controladas, complementando dados existentes em campo.

Referências Bibliográficas

- AGORAMOORTHY, G.; CHEN, F-A.; HSU, M.J. Threat of heavy metal pollution in halophytic and mangrove plants of Tamil Nadu, India. **Environmental Pollution**, v. 155, p. 320-326, 2008.
- ARMSTRONG, W. **Aeration in higher plants**. In: Advances in botanical Research, Vol. 7. Ed. Woolhouse H.W.W. Academic Press, pp. 225-332. 1979.
- ARRIVABENE, H.P. **Morfoanatomia, histoquímica e teor de ferro foliar em *Avicennia schaueriana* Stapft & Leechm, *Laguncularia racemosa* (L.) Gaertn. e *Rhizophora mangle* L. ocorrentes em cinco áreas de manguezal do estado do Espírito Santo**. 130f. Dissertação (Mestrado em Biologia Vegetal) – Programa de Pós-Graduação em Biologia Vegetal da Universidade Federal do Espírito Santo, Vitória, 2011.
- ARRIVABENE, H.P.; Souza, I.C; CÓ, W.L.O.; CONTI, M.M.; WUNDERLIN, D.A.; MILANEZ, C.R.D. Effect of pollution by particulate iron on the morphoanatomy, histochemistry, and bioaccumulation of three mangrove plant species in Brazil. **Chemosphere**, v. 127, pp. 27-34, 2015.
- BALL, M. Mangrove species richness in relation to salinity and waterlogging: a case study along the Adelaide River floodplain, northern Australia. **Global Ecology & Biogeography Letters**, v. 7, n. 1, p. 73-82, 1998.
- BARAKAT, M.A. New trends in removing heavy metals from industrial wastewater. **Arabian Journal of Chemistry**, v. 4, n. 4, p. 361-377, 2011.
- BARBIER, E.B.; HACKER, S.D.; KENNEDY, C.; KOCH, E.W.; STIER, A.C.; SILLIMAN, B.R. The value of estuarine and coastal ecosystem services. **Ecological Monographs**, v. 81, n. 2, p. 169-193, 2011.
- BAYEN, S. Occurrence, bioavailability and toxic effects of trace metals and organic contaminants in mangrove ecosystems: a review. **Environment international**, v. 48, p. 84-101, 2012.
- BERNINI, E.; SILVA, M. A. B.; CARMO, T. M. S.; CUZZUOL, G. R. F. Composição química do sedimento e de folhas das espécies do manguezal do estuário do Rio São Mateus, Espírito Santo, Brasil. **Revista Brasileira de Botânica**, v. 29, n.4, p.689-699, 2006.

- CHENG, H., CHEN, D.T., TAM, N.F.Y., CHEN, G.Z., LI, S.Y., YE, Z.H.. Interactions among Fe^{2+} , S^{2-} , and Zn^{2+} tolerance, root anatomy, and radial oxygen loss in mangrove plants. **Journal of Experimental Botany**, v. 63, p. 2619–2630, 2012b.
- CHENG, H.; JIANG, Z.Y.; LIU, Y.; YE, Z.H.; WU, M.L.; SUN, C.C.; SUN, F.L.; FEI, J.; WANG, Y.S. Metal (Pb, Zn and Cu) uptake and tolerance by mangroves in relation to root anatomy and lignification/suberization. **Tree physiology**, v. 34, n. 6, 2014a.
- CHENG, H.; LIU, Y.; TAM, N.F.Y.; WANG, X.; LI, S.Y.; CHEN, G.Z.; YE, Z.H. The role of radial oxygen loss and root anatomy on zinc uptake and tolerance in mangrove seedlings. **Environmental pollution**, v. 158, n. 5, p. 1189-1196, 2010.
- CHENG, H.; TAM, N.F.Y.; WANG, Y.; Li, S.; CHEN, G.; YE, Z. Effects of copper on growth, radial oxygen loss and root permeability of seedlings of the mangroves *Bruguiera gymnorhiza* and *Rhizophora stylosa*. **Plant and soil**, v. 359, n. 1-2, p. 255-266, 2012a.
- CHENG, H.; WANG, M.; WONG, M.H.; YE, Z. Does radial oxygen loss and iron plaque formation on roots alter Cd and Pb uptake and distribution in rice plant tissues?. **Plant and soil**, v. 375, n. 1-2, p. 137-148, 2014b.
- CLOUGH, B. **Continuing the Journey Amongst Mangroves**. ISME Mangrove Educational Book Series No. 1. International Society for Mangrove Ecosystems (ISME), Okinawa, Japan, and International Tropical Timber Organization (ITTO), Yokohama, Japan. 2013.
- CONNOLLY, E.L.; GUERINOT, M. L. Iron stress in plants. **Genome Biology**, v.3, p. 1024.1–1024.4., 2002.
- COSTANZA, R., D'ARGE, R., GROOT, R. D., FARBER, S., GRASSO, M., HANNON, B., LIMBURG, K.; NAEEM, S.; O'NEILL, R.V.; PARUELO, J.; RASKIN, R.G.; SUTTON, P.; BELT, M.V.D. The value of the world's ecosystem services and natural capital. **Nature**, v. 387, p. 253-260, 1997.
- CRAM, W.J.; TORR, P.G.; ROSE, D.A. Salt allocation and leaf development and leaf fall in mangroves. **Trees**, v. 16, p. 112–119, 2002.
- CUZZUOL, G.R.F.; CAMPOS, A. Aspectos nutricionais na vegetação de manguezal do estuário do Rio Mucuri, Bahia, Brasil. **Revista Brasileira de Botânica**, v. 24, n. 2, p. 227-234, 2001.
- DEFEW, L.H.; MAIR, J.M.; GUZMAN, H.M. An assessment of metal contamination in mangrove sediments and leaves from Punta Mala Bay, Pacific Panama. **Marine Pollution Bulletin**, v. 50, p. 547–552, 2005.

- DU LAING, G.; RINKLEBE, J.; VANDECASTEELE, B.; MEERS, E.; TACK, F.M.G. Trace metal behaviour in estuarine and riverine floodplain soils and sediments: a review. **Science of the Total Environment**, v. 407, n. 13, p. 3972-3985, 2009.
- ELLISON, A.M.; FARNSWORTH, E.J.; MERKT, R.E. Origins of mangrove ecosystems and the mangrove biodiversity anomaly. **Global Ecology and Biogeography**, v. 8, n. 2, p. 95-115, 1999.
- FRANCISCO, A.M.; Díaz, M.; Romano, M.; Sánchez, F. Descripción morfoanatomia de los tipos de glândulas foliares em El mangle blanco *Laguncularia racemosa* L. Gaertn (f.). **Acta Microscopica**, v. 18, n. 3, p. 237-252, 2009.
- GBARUKO B.C.; FRIDAY O.U. Bioaccumulation of heavy metals in some fauna and flora. **International Journal of Environmental Science and Technology**, v.4, n.2, p. 197-202, 2007.
- GIMENO-GARCÍA, E.; ANDREU, V.; BOLUDA, R.. Heavy metals incidence in the application of inorganic fertilizers and pesticides to rice farming soils. **Environmental pollution**, v. 92, n. 1, p. 19-25, 1996.
- GIRI, C.; OCHIENG, E.; TIESZEN, L.L.; ZHU, Z.; SINGH, A.; LOVELAND, T.; MASEK, J.; DUKE, N. Status and distribution of mangrove forests of the world using earth observation satellite data. **Global Ecology and Biogeography**, v. 20, n. 1, p. 154-159, 2011.
- GUHATHAKURTA, H.; KAVIRAJ, A. Heavy metal concentration in water, sediment, shrimp (*Penaeus monodon*) and mullet (*Liza parsia*) in some brackish water ponds of Sunderban, India. **Marine Pollution Bulletin**, v. 40, n. 11, p. 914-920, 2000.
- HOSSAIN, M.A.; PIYATIDA, P.; DA SILVA, J.A.T.; FUJITA, M. Molecular mechanism of heavy metal toxicity and tolerance in plants: central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. **Journal of Botany**, v. 2012, p. 1-37, 2012.
- HSU, M.J.; SELVARAJ, K.; AGORAMOORTHY, G. Taiwan's industrial heavy metal pollution threatens terrestrial biota. **Environmental Pollution**, v. 143, p. 327-334, 2006.
- HWANG, Y-H.; CHEN, S-C. Salt tolerance in seedlings of the mangrove *Kandelia candel* (L.) Druce, Rhizophoraceae. **Botanical Bulletin of Academia Sinica**, v. 36, n. 1, p. 25-31, 1995.
- INSTITUTO BRASILEIRO DE EXECUTIVOS DE FINANÇAS – IBEF. Disponível em: <<http://ibefes.ning.com/page/ibefes-faz-visita-tecnica-a>>. Acesso em: 13 fev. 2011.

- KIM, S.A.; M. L. GUERINOT. Mining iron: Iron uptake and transport in plants. **FEBS Letters**, v. 581, p. 2273–2280, 2007.
- KJERFVE, B.; LACERDA, L.D. **Mangroves of Brazil**. In: LD Lacerda (ed.) Conservation and sustainable utilization of mangrove forest in Latin America and Africa regions, Part I: Latin America, 1st edn. ITTO/ISME, Okinawa, pp 245–272. 1993.
- KOBAYASHI, T.; NISHIZAWA, N.K. Iron uptake, translocation, and regulation in higher plants. **Annual review of plant biology**, v. 63, p. 131-152, 2012.
- LACERDA, L.D.; ABRÃO, J. J. Heavy metal accumulation by mangrove and saltmarsh intertidal sediments. **Revista Brasileira de Botânica**, v. 7, p. 49-52, 1984.
- LACERDA, L.D.; SOUZA, C.M.M.; PESTANA, M.H.D. Trace metals geochemical associations in sediments of a non-contaminated estuary. **Ciência e Cultura**, v. 41, p. 301-304, 1989.
- LEWIS, M.; PRYOR, R.; WILKING, L. Fate and effects of anthropogenic chemicals in mangrove ecosystems: a review. **Environmental pollution**, v. 159, n. 10, p. 2328-2346, 2011.
- LIU, Y.; TAM, N.F.Y.; YANG, J.X.; PI, N.; B, WONG, M.H.; YE, Z.H. Mixed heavy metals tolerance and radial oxygen loss in mangrove seedlings. **Marine Pollution Bulletin**, v. 58, p. 1843–1849, 2009.
- LU, H.; LIU, B.; ZHANG, Y.; YE, J.; YAN, C. Comparing analysis of elements sub-cellular distribution in *Kandelia obovata* between SEM-EDX and chemical extraction. **Aquatic Botany**, v. 112, p. 10-15, 2014.
- MACFARLANE, G.R.; BURCHETT, M.D. Cellular distribution of copper, lead and zinc in the grey mangrove, *Avicennia marina* (Forsk.) Vierh. **Aquatic Botany**, v. 68, p. 45–59, 2000.
- MACFARLANE, G.R.; BURCHETT, M.D. Toxicity, growth and accumulation relationships of copper, lead and zinc in the grey mangrove *Avicennia marina* (Forsk.) Vierh. **Marine Environmental Research**, v. 54, p. 65–84, 2002.
- MACFARLANE, G.R.; BURCHETT, M.D. Zinc distribution and excretion in the leaves of the grey mangrove, *Avicennia marina* (Forsk.) Vierh. Environ. **Experimental Botany**, v. 41, p. 167–175, 1999.
- MACFARLANE, G.R.; PULKOWNIK, A.; BURCHETT, M.D. Accumulation and distribution of heavy metals in the grey mangrove, *Avicennia marina* (Forsk.) Vierh.: biological indication potential. **Environmental Pollution**, v. 123, p. 139–151, 2003.

- MACHADO, W.; SANTELLI, R.E.; CARVALHO, M.F.; MOLISANI, M.M.; BARRETO, R.C.; LACERDA, L.D. Relation of reactive sulfides with organic carbon, iron, and manganese in anaerobic mangrove sediments: implications for sediment suitability to trap trace metals. **Journal of Coastal Research**, v. 24, p. 25-32, 2008.
- MACHADO, W.; SILVA-FILHO, E.V.; OLIVEIRA, R.R.; LACERDA, L.D. Trace metal retention in mangrove ecosystems in Guanabara Bay, SE Brazil. **Marine Pollution Bulletin**, v. 44, p. 1277–1280, 2002.
- MACHADO, W.; GUEIROS, B.B.; LISBOA-FILHO, S.D.; LACERDA, L.D. Trace metals in mangrove seedlings: role of iron plaque formation. **Wetlands Ecology and Management**, v. 13, p. 199–206, 2005.
- MANSANO, V.F.; BARROS, L.A.V. de; ASSUNÇÃO, V.A. *Rhizophoraceae* in **Lista de Espécies da Flora do Brasil**. Jardim Botânico do Rio de Janeiro. Disponível em: <<http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB208>>. Acesso em: 28 Jun. 2015
- MARQUETE, N.; LOIOLA, M.I.B. *Combretaceae* in **Lista de Espécies da Flora do Brasil**. Jardim Botânico do Rio de Janeiro. Disponível em: <<http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB90>>. Acesso em: 28 Jun. 2015
- MARSCHNER, H. **Mineral Nutrition of Higher plants**. New York: Academic Press, 2012.
- MENEZES, N. L. Rhizophores in *Rhizophora mangle* L: an alternative interpretation of so-called "aerial roots". **Anais da Academia Brasileira de Ciências**. v. 78, n. 2, p. 213-226, 2006.
- MORSE, J.W.; RICKARD, D. Peer reviewed: chemical dynamics of sedimentary acid volatile sulfide. **Environmental science & technology**, v. 38, n. 7, p. 131A-136A, 2004.
- NAIDOO, G.; HIRALAL, T.; NAIDOO, Y. Ecophysiological responses of the mangrove *Avicennia marina* to trace metal contamination. **Flora**, v. 209, n. 1, p. 63-72, 2014.
- PARIDA, A.K.; DAS, A.B.; SANADA, Y.; MOHANTY, P. Effects of salinity on biochemical components of the mangrove, *Aegiceras corniculatum*. **Aquatic Botany**, v. 80, n. 2, p. 77-87, 2004.
- PETERS, E.C.; GASSMAN, N.J.; FIRMAN, J.C.; RICHMOND, R.H.; POWER, E.A. Ecotoxicology of tropical marine ecosystems. **Environmental Toxicology and Chemistry**, v. 16, p. 12–40, 1997.
- PI, N.; TAM, N.F.Y.; WU, Y.; WONG, M.H. Root anatomy and spatial pattern of radial oxygen loss of eight true mangrove species. **Aquatic Botany**, v. 90, n. 3, p. 222-230, 2009.

- PROFICE, S.R.; KAMEYAMA, C.; CÔRTEZ, A.L.A.; BRAZ, D.M.; INDRIUNAS, A.; VILAR, T.; PESSOA, C.; EZCURRA, C.; WASSHAUSEN, D. *Acanthaceae* in **Lista de Espécies da Flora do Brasil**. Jardim Botânico do Rio de Janeiro. Disponível em: <<http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB33>>. Acesso em: 28 Jun. 2015
- SADIQ, M.; ZAIDI, T.H. Sediment composition and metal concentrations in mangrove leaves from the Saudi coast of the Arabian Gulf. **Science of the Total Environment**, v. 155, p.1-8, 1994.
- SADOONI, F.N.; AL-SAAD, H. Mangrove-bearing limestone from the Eocene Dammam Formation, Arabian Gulf: implications for the mangrove dispersal controversy. **Carbonates and evaporites**, v. 27, n. 3-4, p. 243-250, 2012.
- SAENGER, P. **Mangrove Ecology, Silviculture and Conservation**. Kluwer Academic Publishers. 2002.
- SAIFULLAH, S.M.; ISMAIL, S.; KHAN S.H.; SALEEM, M. Land Use—Iron Pollution in Mangrove Habitat of Karachi, Indus Delta. **Earth Interactions**, v. 8, p. 1-9, 2004.
- SARANGI, R.K.; KATHIRESAN, K.; SUBRAMANIAN, A.N. Metal concentrations in five mangrove species of the Bhitarkanika, Orissa, east coast of India. **Indian Journal of Marine Species**, v. 31, n. 3, p. 251-253, 2002.
- SCHOLANDER, P. F.; HAMMEL, H. T.; HEMMINGSON, E. A.; GAREY, W. Salt balance in mangroves. **Plant Physiology**, v. 37, p. 722-729, 1962.
- SHERMAN, R.E.; FAHEY, T.J.; HOWARTH, R.W. Soil-plant interactions in a neotropical mangrove forest: iron, phosphorus and sulfur dynamics. **Oecologia**, v. 115, 553-563, 1998.
- SILVA FILHO, E.V.; PAIVA, R.P.; WASSERMAN, J.C.; LACERDA, L.D. Geochemical Characterization of Rain Water Particulate Material on a Coastal Sub-Tropical Region in SE – Brazil. **Journal of the Brazilian Chemical Society**, v. 9, p. 482-486, 1998.
- SILVA, C.A.R.; LACERDA, L.D.; REZENDE, C.E. Metals reservoir in a red mangrove forest. **Biotropica**, v. 22, n. 4, p. 339-345, 1990.
- SOBRADO, M. A. Leaf characteristics and gas exchange of the mangrove *Laguncularia racemosa* as affected by salinity. **Photosynthetica**, v. 43, p. 217-221, 2005.
- SOBRADO, M.A. Influence of external salinity on the osmolality of xylem sap, leaf tissue and leaf gland secretion of the mangrove *Laguncularia racemosa* (L.) Gaertn. **Trees**, v. 18, p. 422–427, 2004.

- SOUZA, I.; BONOMO, M.M.; MOROZESK, M.; ROCHA, L.D.; DUARTE, I.D.; FURLAN, L.M.; ARRIVABENE, H.P.; MONFERRÁN, M.V.; MATSUMOTO, S.T.; MILANEZ, C.R.D.; WUNDERLIN, D.A.; FERNANDES, M.N. Adaptive plasticity of *Laguncularia racemosa* in response to different environmental conditions: integrating chemical and biological data by chemometrics. **Ecotoxicology**, v. 23, n. 3, p. 335-348, 2014a.
- SOUZA, I.C.; MOROZESK, M.; DUARTE, I.D.; BONOMO, M.M.; ROCHA, L.D.; FURLAN, L.M.; ARRIVABENE, H.P.; MONFERRÁN, M.V.; MATSUMOTO, S.T.; MILANEZ, C.R.D.; WUNDERLIN, D.A.; FERNANDES, M.N. Matching pollution with adaptive changes in mangrove plants by multivariate statistics. A case study, *Rhizophora mangle* from four neotropical mangroves in Brazil. **Chemosphere**, v. 108, p. 115-124, 2014b.
- SOUZA, I.C.; ROCHA, L.D.; MOROZESK, M.; BONOMO, M.M.; ARRIVABENE, H.P.; DUARTE, I.D., FURLAN, L.M., MONFERRÁN, M.V.; MATSUMOTO, S.T.; MILANEZ, C.R.D.; WUNDERLIN, D.A.; FERNANDES, M.N. Changes in bioaccumulation and translocation patterns between root and leaf of *Avicennia schaueriana* as adaptive response to different levels of metals in mangrove system. **Marine pollution bulletin**, 2015.
- SOUZA, V.; LORENZI, H. **Botânica sistemática: guia ilustrado para identificação das famílias de fanerógamas nativas e exóticas no Brasil, baseado em APG II**. 2008.
- SPALDING, M. **World atlas of mangroves**. Routledge, 2010.
- SUÁREZ, N.; SOBRADO, M. A. Adjustments in leaf water relations of mangrove (*Avicennia germinans*) seedlings grown in a salinity gradient. **Tree Physiology**, v. 20, p. 277–282, 2000.
- SZEFER, P.; GELDON, J.; ALI, A.A.; OSUNA, F.P.; RUIZ-FERNANDESS, A.C. R.; GALVAN, G. Distribution and association of trace metals in soft tissue and byssus of *Mytella strigata* and other benthic organisms from Mazatlan Harbour, Mangrove Lagoon of the northwest coast of Mexico. **Environment International**, v. 24, n. 3, p. 359-374, 1998.
- TAM, N.F.Y.; WONG, W.S. Spatial variation of heavy metals in surface sediments of Hong Kong mangrove swamps. **Environmental Pollution**, v. 110, p. 195–205, 2000.
- TAM, N.F.Y.; WONG, Y.-S. Accumulation and distribution of heavy metals in a simulated mangrove system treated with sewage. **Hydrobiologia**, v. 352, p. 67–75, 1997.

- TAM, N.F.Y.; YAO, M.W.Y. Normalisation and heavy metal contamination in mangrove sediments. **Science of the Total Environment**, v. 216, p. 33-39, 1998.
- THIBODEAU, F.R.; NICKERSON, N.H. Differential oxidation of mangrove substrate by *Avicennia germinans* and *Rhizophora mangle*. **American Journal of Botany**, v. 73, n. 4, p. 512-516, 1986.
- THOMINE, S.; VERT, G. Iron transport in plants: better be safe than sorry. **Current opinion in plant biology**, v. 16, n. 3, p. 322-327, 2013.
- TOMLINSON, P. B. **The botany of mangroves**. New York: Cambridge University Press, 1994.
- TRIPATHI, R.D.; TRIPATHI, P.; DWIVEDI, S.; KUMAR, A.; MISHRA, A.; CHAUHAN, P.S.; NORTON G.J.; NAUTIYAL, C.S. Roles for root iron plaque in sequestration and uptake of heavy metals and metalloids in aquatic and wetland plants. **Metallomics**, v. 6, n. 10, p. 1789-1800, 2014.
- VALIELA, I.; BOWEN, J.L.; YORK, J.K. Mangrove Forests: One of the World's Threatened Major Tropical Environments At least 35% of the area of mangrove forests has been lost in the past two decades, losses that exceed those for tropical rain forests and coral reefs, two other well-known threatened environments. **Bioscience**, v. 51, n. 10, p. 807-815, 2001.
- VO, Q.T.; KUENZER, C.; VO, Q.M.; MODER, F.; OPPELT, N. Review of valuation methods for mangrove ecosystem services. **Ecological Indicators**, v. 23, p. 431-446, 2012.
- WALTERS, B.B.; RÖNNBÄCK, P.; KOVACS, J.M.; CRONA, B.; HUSSAIN, S. A.; BADOLA, R.; PRIMAVERA, J.H.; BARBIER, E.; DAHDOUNH-GUEBAS, F. Ethnobiology, socio-economics and management of mangrove forests: a review. **Aquatic Botany**, v. 89, n. 2, p. 220-236, 2008.
- WENG, B.; XIE, X.; WEISS, D.J.; LIU, J.; LU, H.; YAN, C. *Kandelia obovata* (S., L.) Yong tolerance mechanisms to cadmium: subcellular distribution, chemical forms and thiol pools. **Marine pollution bulletin**, v. 64, n. 11, p. 2453-2460, 2012.
- WOODROFFE, C. **Mangrove sediments and geomorphology**. In: Robertson AI, Alongi DM (eds.) Coastal and estuarine studies, Vol 41. American Geophysical Union, Washington, pp 7–41. 1992.

- YE, Y.; TAM, N.F.Y; LU, C.Y.; WONG, Y.S. Effects of salinity on germination, seedling growth and physiology of three salt secreting mangrove species. **Aquatic Botany**, v. 83, p. 193–205, 2005.
- ZHOU, Y.W.; PENG, Y.S.; LI, X.L.; CHEN, G.Z. Accumulation and partitioning of heavy metals in mangrove rhizosphere sediments. **Environmental Earth Sciences**, v. 64, n. 3, p. 799-807, 2011.
- ZHOU, Y-W.; ZHAO, B.; PENG, Y-S; CHEN, G-Z. Influence of mangrove reforestation on heavy metal accumulation and speciation in intertidal sediments. **Marine Pollution Bulletin**, v. 60, p. 1319-1324, 2010.