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5 **Influence of salinity on hydrogen isotope fractionation in *Rhizophora***
6 **mangroves from Micronesia**
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Abstract

Hydrogen isotope ratios ($^2\text{H}/^1\text{H}$ or $\delta^2\text{H}$) of plant leaf waxes typically covary with those of precipitation, and are therefore used as a proxy for past hydrologic variability. Mangroves present an important exception to this relationship, as salinity can strongly influence ^2H fractionation in leaf lipids. To better understand and calibrate this effect, $\delta^2\text{H}$ values of taraxerol and *n*-alkanes were measured in the leaves of *Rhizophora* spp. (red mangroves) from three estuaries and four brackish lakes on the Micronesian islands of Pohnpei and Palau, and compared to the $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of leaf water, xylem water and surface water. Net ^2H discrimination between surface water and taraxerol increased by $0.9 \pm 0.2\text{‰}$ per part per thousand (ppt⁻¹) over a salinity range of 1-34 ppt. Xylem water was always depleted in ^2H relative to surface water, and the magnitude of this depletion increased with salinity, which is most likely due to a combination of greater ^2H discrimination by roots during water uptake and opportunistic use of freshwater. Changes in the ^2H content of xylem water can account for up to 43% of the change in net taraxerol fractionation with salinity. Leaf water isotopes were minimally enriched relative to xylem water and there was not significant variability in leaf water enrichment with salinity, which is consistent with a Péclet-modified Craig-Gordon model of leaf water enrichment. As leaf water enrichment is therefore unlikely to be responsible for increased $^2\text{H}/^1\text{H}$ fractionation in mangrove leaf lipids at elevated salinities, the majority of this signal is most likely explained either by changes in biosynthetic fractionation in response to salt stress or by salinity influenced changes in the timing of water uptake and lipid synthesis.

1. INTRODUCTION

Hydrogen isotope ratios of plant leaf waxes are increasingly used as a proxy for past hydrologic variability (Sachse et al., 2012, and sources therein). This proxy is founded on the assumption that the hydrogen in leaf waxes and other plant lipids is derived from environmental water, and the observation that the $\delta^2\text{H}_{\text{Lipid}}$ values ($\delta^2\text{H} = ((^2\text{H}/^1\text{H})_{\text{sample}}/(^2\text{H}/^1\text{H})_{\text{VSMOW}}) - 1$; $\delta^2\text{H} = \delta\text{D}$) are highly correlated with local $\delta^2\text{H}_{\text{Precipitation}}$ values (Huang et al., 2004; Sachse et al., 2004; Hou et al., 2008; Polissar and Freeman, 2010; Sachse et al., 2012). $\delta^2\text{H}_{\text{Precipitation}}$ values are determined by environmental parameters, including temperature, precipitation rate, and moisture source (Dansgaard 1964; Craig and Gordon, 1965; Gat, 1996).

Leaf waxes and other lipids are typically very depleted in ^2H relative to the plant's source water (Sessions et al., 1999; Sauer et al., 2001; Chikaraishi and Naraoka, 2003; Sachse et al., 2012). The difference between $\delta^2\text{H}_{\text{Lipid}}$ values and $\delta^2\text{H}_{\text{Water}}$ values is expressed using the net fractionation factor, $\alpha_{\text{Lipid-Water}} = (^2\text{H}/^1\text{H})_{\text{Lipid}}/(^2\text{H}/^1\text{H})_{\text{Water}}$. The magnitude of $\alpha_{\text{Lipid-Water}}$ is sensitive to variables such as plant type (Luo et al., 2006; Smith and Freeman, 2006; Hou et al., 2007), relative humidity (Feakins and Sessions, 2010; Kahmen et al., 2013a, b; Tipple et al., 2014), and light levels (Luo and Yang, 2008; Yang et al., 2009; Yang et al., 2011). In order to reliably use sedimentary $\delta^2\text{H}_{\text{Lipid}}$ values to infer information about past changes in climate, it is important to better understand causes of variability in $\alpha_{\text{Lipid-Water}}$, which can result in changes to $\delta^2\text{H}_{\text{Lipid}}$ values that are not solely driven by changes in $\delta^2\text{H}_{\text{Precipitation}}$ values.

Of particular importance for coastal sediments in the tropics and subtropics, the salinity of environmental water can influence $\alpha_{\text{Lipid-Water}}$ (Romero and Feakins, 2011; Ladd and Sachs, 2012). Salt tolerant mangrove trees are the dominant plant type in the intertidal zone of many low-latitude coastlines, and form highly productive ecosystems. As much as 30% of the total

terrestrial carbon accumulating in modern subtropical and tropical coastal sediments is mangrove derived (Alongi and Mukhopadhyay, 2014). With so much of the plant material in such sediment coming from a mangrove source, it is important to fully determine the effect of salinity on hydrogen isotope fractionation.

The influence of salinity on $\alpha_{\text{Lipid-Water}}$ in leaf wax *n*-alkanes produced by *Avicennia marina* (gray mangroves) was previously investigated along a salinity gradient of 6-35 practical salinity units (PSU) in the Brisbane River Estuary in Queensland, Australia (Ladd and Sachs, 2012). A decrease in $\alpha_{\text{Lipid-Water}}$ of $1.5\text{‰} \pm 0.3\text{‰}$ PSU⁻¹ was observed for both *n*C₃₁- and *n*C₃₃-alkanes. Several hypotheses were proposed that could explain this relationship: (1) preferential exclusion of ²H during water uptake, (2) increased compatible solute production, preferentially using relatively enriched H⁺ from the pentose phosphate cycle, and leaving relatively depleted H⁺ from recent photosynthesis available for incorporation into lipids, or (3) increased salt secretion at high salinity, which could decrease the $\delta^2\text{H}$ value of leaf water ($\delta^2\text{H}_{\text{LW}}$) by creating a layer of isotopically depleted water hydrating the salt on the surface of leaves. Because intermediate xylem water and leaf water pools were not analyzed in the Brisbane River study, it was not possible to definitively exclude or support any of these hypotheses.

The results of the Brisbane River calibration were significant for two reasons. First of all, they demonstrated a potential pitfall of using $\delta^2\text{H}$ values of generic leaf waxes to reconstruct climate in coastal tropical settings where mangroves are present. Leaf wax *n*-alkanes are ubiquitous in vascular plants, and it is impossible to distinguish *n*C₃₁-alkanes in a sediment sample from a mangrove or terrestrial plant source. If local climate became more arid, one would expect the $\delta^2\text{H}_{\text{Wax}}$ values of terrestrial plants to increase, since $\delta^2\text{H}_{\text{Precipitation}}$ values increase with

decreasing precipitation amount in the tropics (Dansgaard 1964; Gat 1996; Kurita et al., 2009; Conroy et al., 2013).

Although mangrove trees in the region would also be using isotopically enriched water, the salinity of that water would be expected to increase as less freshwater was delivered through rain. Since increased salinity results in more net discrimination against ^2H during lipid synthesis by mangroves (Ladd and Sachs, 2012), the $\delta^2\text{H}_{\text{Wax}}$ from mangroves could actually decrease in more arid conditions. In Brisbane, for example, *Avicennia marina* growing at 35 PSU produced leaf wax lipids that were depleted by ~50‰ relative to trees growing at 6 PSU, even though the $\delta^2\text{H}_{\text{Water}}$ was enriched by ~15‰ (Ladd and Sachs, 2012). Opposing $\delta^2\text{H}_{\text{Wax}}$ responses to the same environmental shift would result in a muted signal in sediments that contain the molecular remains of both plant types. Relatively small $\delta^2\text{H}_{\text{Wax}}$ variability has in fact been observed in many coastal tropical records and could reflect this opposing hydrogen isotopic response (Tierney et al., 2010; Smittenberg et al., 2011).

While the inverse relationship between salinity and isotope fractionation in mangroves complicates the interpretation of $\delta^2\text{H}_{\text{Wax}}$ values in low-latitude coastal settings, it also forms the basis for quantitative reconstructions of both $\delta^2\text{H}_{\text{Water}}$ values and salinity. If the relationship between salinity and $\alpha_{\text{Lipid-Water}}$ observed in leaf waxes from the Brisbane River holds true for more mangrove-specific biomarkers, it will be possible to quantitatively reconstruct past salinity and $\delta^2\text{H}_{\text{Water}}$ values by measuring both $\delta^2\text{H}$ and $\delta^{13}\text{C}$ values in a single mangrove lipid, or by measuring $\delta^2\text{H}$ values of co-occurring lipids from phytoplankton and mangroves (Ladd and Sachs, 2012; Ladd and Sachs, 2013). The first of these strategies relies on the observation that $\delta^{13}\text{C}$ values of mangrove *n*-alkanes increase with increasing salinity, resulting in an independent measure of past salinity (Ladd & Sachs, 2013). The second approach is based on the observation

that microalgae fractionate hydrogen isotopes less as salinity increases (Schouten et al, 2006; Sachse and Sachs, 2008; Sachs and Schwab, 2011; Chivall et al., 2014; Nelson and Sachs, 2014).

Required for both of these approaches is a robust calibration of the relationship between salinity and $\alpha_{\text{Lipid-Water}}$ for a mangrove specific lipid biomarker. Because the pentacyclic triterpenoid taraxerol is produced in high abundance by *Rhizophora* spp. mangroves (Killops and Frewin, 1994; Koch et al., 2003; Versteegh et al., 2004), and because it is relatively refractory and well preserved in sediment (Koch et al., 2003; Koch et al., 2005), it is a promising target compound. The primary goal of the present study was to assess the influence of salinity on net hydrogen isotope fractionation during the biosynthesis of taraxerol.

A related goal was to better understand the mechanisms by which isotopic fractionation occurs during lipid synthesis in mangroves. The only published study on the relationship between salinity and net H isotope fractionation in mangroves (Ladd and Sachs, 2012) presented $\delta^2\text{H}$ data from leaf wax *n*-alkanes and surface water (SW). This allowed $\alpha_{\text{Lipid-SW}}$ to be calculated, but left the fractionation associated with several intermediate steps unconstrained. These steps include potential discrimination against ^2H during water uptake (that is, depletion of xylem water (XW) relative to surface water or $\alpha_{\text{XW-SW}}$), which is uncommon in most higher plants, but demonstrated to occur in halophytes (Lin and Sternberg, 1993; Ellsworth and Williams, 2007), enrichment of leaf water (LW) due to transpiration ($\alpha_{\text{LW-XW}}$; Dawson et al., 2002; Kahmen et al., 2008), and biosynthetic fractionation during photosynthesis and lipid synthesis ($\alpha_{\text{Lipid-LW}}$). Analyzing $\delta^2\text{H}_{\text{XW}}$ and $\delta^2\text{H}_{\text{LW}}$ values, in addition to environmental water and lipids, is an effective method for determining fractionation associated with these intermediate steps (Feakins and Sessions, 2010; Romero and Feakins, 2011; Kahmen et al., 2013b; Tipple et al., 2013).

2. METHODS

2.1. Site description

This study focused on *Rhizophora* mangroves growing in two western Pacific sites: the island of Pohnpei (7 °N, 158 °E) in the Federated States of Micronesia, and the rock islands of Palau (7 °N, 134 °E) (Figure 1). In Pohnpei, samples were collected from the banks of three estuaries, the Kamar (July 2011), the Sapwalap (August 2012) and the Soundau (August 2012) (Figure 1). Trees were sampled at five sites along the Kamar estuary, spanning a salinity range of 1-30 parts per thousand (ppt), at eleven sites on the Sapwalap, spanning a salinity range of 1-34 ppt and at seven sites along the Soundau, spanning a salinity range of 4-33 ppt (Table 1). Salinity values are reported in ppt here, as they were measured with a refractometer (VeeGee Scientific, Kirkland, WA) with accuracy of ± 1 ppt. Multiple salinity measurements were made at each site over the sampling period, and at additional sites along each estuary at different points in the tidal cycle.

Salinity was highly correlated with distance from the river mouth in each estuary (0.90 in Kamar, 0.78 in Sapwalap, and 0.93 in Soundau) and the line of best fit relating measured salinities to distance from the river mouth was used to determine the mean salinities used in all figures and Table 1, which was deemed preferable to using the measured salinity at a single time point in the tidal cycle. The standard error of the regression between salinity and distance from the river mouth was 5.0 for Kamar, 4.4 for Sapwalap, and 2.8 for Soundau. These values are used for the salinity error bars in all tables and figures.

In Palau, trees were sampled in September and October of 2013 from Little Crocodile Lake and Big Crocodile Lake on Mecherchar Island and from Long Lake on Ngeruktabel Island

(Figure 1). These marine lakes have been described in detail (Hamner et al., 1982; Hamner and Hamner, 1998). They are located in karst terrain and are connected at depth to the ocean through fissures in the surrounding bedrock. While dozens of lakes exist in the rock islands, *Rhizophora* is not present at the majority of them. The lakes for this study were chosen to represent the widest possible salinity range where *Rhizophora* is present.

Salinity in the highly stratified surface water in Little Crocodile Lake has been 8 ± 2 ppt since monitoring began in the 1970's, while surface water salinity in Big Crocodile Lake has been 20 ± 3 ppt (Hamner and Hamner, 1998; Coral Reef Research Foundation). Surface water salinity in these lakes is constant through tidal cycles, but varies with amount of rainfall (Hamner and Hamner, 1998). Unlike the other two lakes, Long Lake is connected to the lagoon by a surface channel, and is not highly stratified. Salinity in this lake is near that of the local lagoon water, averaging 32 ± 1 ppt. In addition to the three lakes, samples were also collected from fringing mangroves in the lagoon on the south coast of Koror, where salinity was 33 ± 1 ppt at two sites and 29 ± 1 ppt at a third.

4 mL of water was collected in a screw cap vial at each site for isotopic analysis. When possible, multiple water samples were collected from a site at different points in the tidal cycle to evaluate the range of surface salinities at individual sample locations along the estuaries.

2.2. Leaf and stem sampling

Leaves and stems were collected from as many as three species of *Rhizophora* at each site. *R. apiculata*, *R. stylosa*, and *R. mucronata* were present and sampled in Pohnpei. When two species were present at the same location, both were sampled. Typically *R. stylosa* and *R. mucronata* were found at the saltier sites and *R. apiculata* was found at the fresher sites, with *R.*

apiculata present through a larger salinity range. In Palau, *R. apiculata* was sampled at Little Crocodile Lake and at one lagoon site. *R. mucronata* was sampled at Big Crocodile Lake, Long Lake, and two lagoon sites.

Stems for xylem water analyses were collected from sun exposed branches ~1 m above the lowest branches on the tree. In Palau, all of these samples came from the north shore of the lake and were south facing branches. ~5 cm of woody stem was collected and stored in an 11 mL glass screw cap vial.

Leaves for leaf water analyses were collected from the same branch as the xylem water samples. Sampled leaves were second from the distal end of the branch, representing the youngest, fully mature leaves on the branch. These leaves were also stored in 11 mL glass screw cap vials.

Leaves for lipids were collected from several additional branches. In Pohnpei, five leaves were collected from different sun exposed branches, ~1 m above the lowest branches on the tree. In Palau, one leaf each was collected from the south, west, and east aspects of the tree, and one leaf was collected from the most shaded interior branch present on the tree (trees on the north shore typically did not have any leaves on their north sides, as this aspect was totally shaded). All leaves for lipid analyses were the youngest fully mature leaf on their respective branches. All leaf and stem samples were placed on ice in the field and then stored at -20 °C prior to processing.

2.3. Leaf water and xylem water extraction

Leaf and xylem water samples were cryogenically extracted on a vacuum line. Samples were heated to 80 °C while attached to a glass U-tube that was submerged in liquid Nitrogen.

The sealed line was kept at a pressure below 60 mTorr for at least two hours. After this time, the U-tubes were removed from the liquid N₂, sealed, and the collected water was allowed to thaw. The water was then filtered through a 0.45 µm PTFE filter to remove any particulate material and transferred to 2 mL screw cap vials.

2.4. Water $\delta^2\text{H}$ and $\delta^{18}\text{O}$ analyses

Leaf and xylem water samples were analyzed by a Thermal Conversion Elemental Analyzer (TC/EA) coupled to a Delta V Isotope Ratio Mass Spectrometer (IRMS; Thermo Scientific, Waltham, MA). Each sample and standard was injected six times in sequence. In order to avoid any memory effects from the previous analysis, the first three injections of each sample were discarded. Thermo Isodat 3.0 software normalized raw isotopic ratios to the VSMOW (Vienna Standard Mean Ocean Water) scale using pulses of reference gas before and after the sample injection. Additionally, two in-house water standards of known isotopic composition were analyzed after every six samples throughout each sequence. The depleted standard had a $\delta^2\text{H}$ value of -77.44‰ and a $\delta^{18}\text{O}$ value of -11.00‰, while the enriched standard had a $\delta^2\text{H}$ value of 44.65‰ and a $\delta^{18}\text{O}$ value of 4.18‰. The slope and intercept of the linear regression between the measured and known δ values of these standards was used to further normalize sample $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values and to correct for any instrumental drift throughout the course of the sequence. An additional laboratory standard ($\delta^2\text{H} = 0.53\text{‰}$; $\delta^{18}\text{O}$ value of -0.83‰) was analyzed after every 11 samples. This standard was not used for any of the isotopic corrections, but was used to assess instrument performance over time and the accuracy of the isotope corrections. Average absolute offset from the known isotopic value of this standard was

1.0‰ for $\delta^2\text{H}$ and 0.3‰ for $\delta^{18}\text{O}$. Average precision of triplicate sample injections was 0.80‰ for $\delta^2\text{H}$ and 0.2‰ for $\delta^{18}\text{O}$.

Surface water $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values were measured by a Picarro Cavity Ring Down Spectroscopy (CRDS) L2130i Water Isotope Analyzer. Measurements were normalized to the VSMOW scale using three laboratory standards of known isotopic composition. The three standards were analyzed between every six samples throughout the run. All samples and standards were injected six times, and the first three measurements were discarded in order to avoid any memory effects from the previous analysis. Average precision of triplicate analyses was 0.2‰ for $\delta^2\text{H}$ and 0.05‰ for $\delta^{18}\text{O}$.

2.5. Leaf water model

Modeled $\delta^2\text{H}$ values of leaf water ($\delta^2\text{H}_{\text{MLW}}$) were calculated using a Péclet-Modified Craig-Gordon model of leaf water enrichment adapted from Kahmen et al. (2011). The Craig-Gordon model of water enrichment is:

$$\Delta^2H_e = \varepsilon^+ + \varepsilon_k + (\Delta^2H_{\text{WV}} - \varepsilon_k) * (e_a/e_i) \quad (1)$$

where Δ^2H_e is evaporative enrichment of leaf water relative to xylem water at the site of evaporation, ε^+ is equilibrium fractionation between liquid water and water vapor, ε_k is kinetic fractionation associated with diffusion of water vapor from within the leaf to the atmosphere, Δ^2H_{WV} is the difference between water vapor $\delta^2\text{H}$ and $\delta^2\text{H}_{\text{XW}}$, and e_a/e_i is the ratio of ambient to intercellular vapor pressures (Craig and Gordon, 1965; Dongman et al., 1974). The ratio of ambient to intercellular vapor pressure is dependent on relative humidity, air temperature and

stomatal conductance (Buck, 1981). Relative humidity and air temperature were estimated from monthly averages for each site (www.nws.noaa.gov). Following observations in cultured *R. mangle* (Lin and Sternberg, 1992), stomatal conductance was estimated at $0.45 \pm 0.15 \text{ mol/m}^2\text{s}$ for salinity ≤ 15 ppt. A 1 ppt increase in salinity above 15 ppt corresponded to a $0.015 \text{ mol/m}^2\text{s}$ decrease in stomatal conductance. The $\delta^2\text{H}$ values of water vapor were calculated assuming equilibrium with precipitation during the study period. Each Pohnpei sampling campaign lasted no longer than 5 days, so the average precipitation isotopes from the entire time period were used to calculate equilibrium water vapor isotopes. Palau sampling took place over one month, and the water isotopes from the three nearest rain events were used to calculate equilibrium water vapor isotope values for Palau samples.

Following Kahmen et al. (2011), the Craig-Gordon model was modified to account for back diffusion of isotopically enriched water away from the site of evaporation using a Péclet effect ($\rho = L_M E / CD$, where L_M is the mixing path length, E is the transpiration stream, C is the molar concentration of water, and D is the diffusivity of $^1\text{H}^2\text{HO}$). L_M was set at $60.0 \pm 20.0 \text{ mm}$ for all leaves, based on previous observations from *R. mangle* in Florida (Ellsworth et al., 2013). The Péclet effect was used to calculate bulk leaf water enrichment, $\Delta^2\text{H}_{\text{LW}}$, as follows:

$$\Delta^2\text{H}_{\text{LW}} = \frac{\Delta^2\text{H}_e(1 - e^{-\rho})}{\rho} \quad (2)$$

Finally, $\delta^2\text{H}_{\text{LW}}$ was calculated as:

$$\delta^2\text{H}_{\text{LW}} = (\Delta^2\text{H}_{\text{LW}} + \delta^2\text{H}_{\text{XW}}) + (\Delta^2\text{H}_{\text{LW}} * \delta^2\text{H}_{\text{XW}}) / 1000 \quad (3)$$

In order to account for multiple sources of uncertainty in both the environmental and plant physiological parameters, the model was run in a 1000 iteration Monte Carlo simulation that included the uncertainties associated with all estimated and measured values. The Matlab script used to run the Monte Carlo simulation is included in the Electronic Annex.

2.6. Lipid extraction and purification

Intact whole leaves from each tree from the Pohnpei estuaries were selected for analysis. From Palau sites, 4 leaves from different aspects of the tree were combined to form one sample. Leaves were rinsed with DI water to remove debris, cut into small (~0.5 cm) pieces using solvent-washed scissors, freeze dried, and ground into a homogenous powder. Lipids from ~0.5 g of dry leaf were extracted using accelerated solvent extraction (ASE-200, Dionex Corp., Sunnyvale, CA, USA) with 9:1 dichloromethane:methanol (DCM:MeOH) 100 °C and 1500 psi for 3 x 5 min static cycles.

An aliquot of the resulting *Rhizophora* total lipid extract (~10 mg) was purified using silica gel column chromatography. Hydrocarbons were eluted with 8 mL hexane, sterols, alcohols and triterpenols with 8 mL 4:1 hexane:ethyl acetate and remaining compounds with 4 mL MeOH.

The alcohol fraction was acetylated at 70 °C for 30 minutes with 25 µL of acetic anhydride of known $\delta^2\text{H}$ composition in 25 µL of pyridine. Lipids in the alcohol fraction were quantified by Gas Chromatography – Mass Spectrometry (GC-MS) using an Agilent (Santa Clara, CA, USA) 6890N gas chromatograph interfaced to an Agilent 5975 quadrupole mass selective detector. Quantification of taraxerol was performed by comparing integrated peak areas with a known amount of 5 α -cholestane that was added to the sample just prior to GC-MS

analysis. The *n*-alkanes were quantified with a GC instrument equipped with flame ionization detection (GC-FID) (Agilent, Santa Clara, CA, USA), following conditions described previously (Ladd & Sachs, 2012). These quantifications were used to determine an appropriate amount of solvent in which to dissolve the sample so that it would have a concentration of ~250 ng/μL (taraxerol samples) or ~100 ng/ μL (*n*-alkanes) when analyzed by Gas Chromatography – Isotope Ratio Mass Spectrometry (GC-IRMS).

2.7. Lipid $\delta^2\text{H}$ analyses

The $\delta^2\text{H}$ values of taraxerol, *n*C₂₉- and *n*C₃₁-alkanes were measured by GC-IRMS on a Thermo DELTA V PLUS system (Thermo Scientific, Waltham, MA, USA). The gas chromatograph (Trace Ultra, Thermo) was equipped with a split-splitless injector operated in splitless mode at 320 °C, a TRIPLUS autosampler (Thermo Scientific), and a VF-17ms capillary column (60 m x 0.25 mm x 0.25 μm, Agilent). For taraxerol analyses, the GC was heated from 120 °C to 260 °C at 20 °C/min, then at 1 °C/min to 300 °C, at 20 °C/min to 325 °C and then held at 325 °C for 20 min. For *n*-alkane analyses, the GC was heated from 120 °C to 250 °C at 20 °C/min, then at 6 °C/min to 325 °C and then held at 325 °C for 12 min. Helium was used as the carrier gas at a constant flow of 1.1 mL/min. Compounds were pyrolyzed in a ceramic reactor at 1400 °C. 1 μL of sample was injected along with 0.5 μL of a mixture of *n*-alkanes of known isotopic composition (A. Schimmelmann, Indiana University, Bloomington, Indiana). For taraxerol analyses, this mixture included *n*C₂₆-, *n*C₂₈-, *n*C₃₂-, *n*C₃₄- and *n*C₄₁-alkanes. For *n*-alkane analyses, the co-injection standards were *n*C₂₁- and *n*C₂₃-alkanes. At the beginning and end of a sequence, as well as after every 4-6 sample injections, a mixture of additional *n*-alkane standards of known isotopic composition was analyzed with the co-injection standards, in place

of a sample. For taraxerol sequences, this external standard was nC_{38} -alkane, which elutes within 1 minute of taraxerol using these GC conditions. For n -alkane sequences, nC_{28} -, nC_{32} - and nC_{34} -alkanes were used as external standards.

Thermo ISODAT software V.2.5 was used to control instrumentation. The slope and intercept of the relationship between measured and known δ^2H values of the n -alkane standards were used to calculate corrected δ^2H values. Offsets between corrected and known δ^2H values of the standards were used to further correct for drift, retention time, and peak area on a sequence-by-sequence basis. Each sample was measured in triplicate. Reported analytical uncertainties are the standard error in the mean of the isotope standards, or the standard deviation of the triplicate sample analyses (whichever value was higher).

The H_{3+} factor was determined each day using pulses of a reference gas of varying heights and was 4.6 ± 0.7 when analyzing Kamar samples in April, 2012, 4.2 ± 0.3 when analyzing Soundau and Sapwalap samples in Nov/Dec, 2012, and 1.71 ± 0.01 when analyzing Palau samples in Feb/Mar 2014.

2.8. Statistics

Statistical analyses were conducted using PRISM software (Graphpad Software Inc., La Jolla, CA). Relationships between fractionation factors and salinity, and modeled leaf water to measured leaf water, were determined using ordinary least squares regression. Regression lines and 95% confidence intervals are only shown on data sets where the slope of the regression was significantly different from 0 at the $p < 0.05$ level. Differences between the slopes of various regressions were assessed using a two-tailed test of the null hypothesis that both slopes are equal. If slopes were not significantly different ($p > 0.05$), the difference between the y-intercepts were

assessed using a pooled slope for all data. The results of all linear regression analyses, whether they produced significant correlations or not, are presented in Table 2.

3. RESULTS

3.1. Relationship between salinity and $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of different water pools

Surface water isotopes ($\delta^2\text{H}_{\text{SW}}$ and $\delta^{18}\text{O}_{\text{SW}}$) in all three estuaries and in the Palau lakes were highly correlated with salinity (Figure 2; Table 2). The slope of the relationship between salinity and both $\delta^2\text{H}_{\text{SW}}$ and $\delta^{18}\text{O}_{\text{SW}}$ was significantly steeper in Palau than in Pohnpei ($p < 0.0001$) (Figure 2; Table 2).

Both isotopes of xylem water ($\delta^2\text{H}_{\text{XW}}$ and $\delta^{18}\text{O}_{\text{XW}}$) in *Rhizophora* were positively correlated with salinity in both Pohnpei and Palau (Figure 2; Table 2; Table S1). However, the slopes of these relationships were shallower than those between the corresponding surface water isotopes and salinity (Figure 2; Table 2).

Leaf water isotopes ($\delta^2\text{H}_{\text{LW}}$ and $\delta^{18}\text{O}_{\text{LW}}$) of *Rhizophora* increased with salinity, but the relationships were much weaker (Figure 2; Table 2; Table S1). This relationship was only significant in the case of Pohnpei (Figure 2; Table 2).

Modeled $\delta^2\text{H}_{\text{LW}}$ was correlated with measured values ($R^2 = 0.60$; $p < 0.0001$) (Figure 3). The slope of the relationship between modeled and measured leaf water isotopes was close to unity (1.1 ± 0.2) and the y-intercept was close to 0 (-2 ± 3) (Figure 3). Average 1σ uncertainties associated with predicted values were 2‰ for $\delta^2\text{H}_{\text{LW}}$.

3.2. Relationship between salinity and ^2H and ^{18}O fractionation among different water pools

The fractionation factor between surface water and xylem water, $\alpha^2_{\text{XW-SW}}$, was always $\leq$ 1.0 and decreased with salinity (Figures 4a and 4b; Table S1). The correlation between $\alpha^2_{\text{XW-SW}}$ and salinity was moderate for Pohnpei and not significant in Palau (Figures 4a and 4b; Table 2). The fractionation factor between the xylem water and the leaf water, $\alpha^2_{\text{LW-XW}}$, was not correlated with salinity at either site (Figures 4c and 4d; Table 2).

For oxygen isotopes, the fractionation factor between surface water and xylem water, $\alpha^{18}_{\text{XW-SW}}$, was near 1.0 and decreased slightly with salinity (Figures S1a and S1b; Table S1). Again, the correlation between $\alpha^{18}_{\text{XW-SW}}$ and salinity was moderate in Pohnpei and not significant in Palau (Figures S1a and S1b; Table 2). There was essentially no trend between $\alpha^{18}_{\text{LW-XW}}$ and salinity (Figures S1c and S1d; Table 2).

3.3. Relationship between salinity and ^2H fractionation between mangrove leaf lipids and different water pools

The apparent fractionation factor between taraxerol and surface water, $\alpha_{\text{Tarax-SW}}$, decreased with increasing salinity in both Pohnpei and Palau (Figures 5a and 5b; Table 2; Table S1). The slope and intercept were not statistically different between the two sites (Table 2). The linear regression describing the relationship between salinity and $\alpha_{\text{Tarax-SW}}$ for the pooled data set is $\alpha_{\text{Tarax-SW}} = (-0.9 \pm 0.2\text{‰}) * S + 0.814 \pm 0.006$. Separate linear regression analyses of the three species (*R. apiculata*, *R. mucronata*, *R. stylosa*) included in the sample set did not yield statistically different results, and the regression shown in Figure 5 are for the pooled data from all species. The apparent fractionation factor between taraxerol and leaf water, $\alpha_{\text{Tarax-LW}}$, was not correlated with salinity (Table 2).

The apparent fractionation factor between *Rhizophora* nC_{29} -alkanes and salinity, $\alpha_{nC_{29}\text{-SW}}$ also declined with increasing salinity (Figures 5c and 5d), although this result was not statistically significant for Palau (Figure 5d; Table 2), where nC_{29} -alkanes were not measured in several leaves owing to low concentrations. There were ample nC_{31} -alkanes in all leaves from Palau, and the negative correlation between $\alpha_{nC_{31}\text{-SW}}$ and salinity was significant here, as well as in Pohnpei (Figures 5e and 5f; Table 2). One outlier was removed from the Pohnpei nC_{31} -alkane analyses. This sample was from a tree where most of the leaves had moss growing on their surface, which was not present at any other sites. Although the sampled leaves did not have this moss present at the time of sampling, they had virtually no nC_{29} -alkanes, anomalously low nC_{31} -alkane concentrations, and anomalously low nC_{31} -alkane δ^2H values.

Salinity and $\alpha_{nC_{29}\text{-LW}}$ were negatively correlated in Pohnpei (Table 2). In Palau, these two variables were not correlated (Table 2). Salinity and $\alpha_{nC_{31}\text{-LW}}$ were not significantly correlated at either site (Table 2).

4. DISCUSSION

4.1. Water sources and discrimination against 2H during water uptake in *Rhizophora* mangroves

In most higher plants, xylem water isotope (δ^2H_{XW} and $\delta^{18}O_{XW}$) values are identical to those of the environmental water used by the plant, and can be used to determine water sources, since apoplastic water uptake by roots is a bulk process that does not result in isotopic fractionation (White et al., 1985; Dawson and Ehleringer, 1991; Walker and Richardson, 1991; Dawson et al., 2002). In the *Rhizophora* trees studied here, δ^2H_{XW} values were typically depleted relative to δ^2H_{SW} (Figures 2 and 4; Table S1). This implies that surface water was not the only

water source for these plants and/or that the roots of these *Rhizophora* trees discriminate against water molecules containing ^2H during water uptake.

Previous studies indicate that mangroves and other salt tolerant plants discriminate against ^2H by as much as 11‰ during water uptake, but not against ^{18}O (Lin and Sternberg, 1993; Ellsworth and Williams, 2007). As such, $\delta^{18}\text{O}_{\text{XW}}$ values have been used to identify mangroves' source water (Lin and Sternberg, 1994; Ewe et al., 2007; Lambs et al., 2008; Wei et al., 2013; Santini et al., 2015). In the estuaries in Pohnpei, the deviation of $\alpha^{18}_{\text{XW-SW}}$ from 1.000 was less than 0.001 (or 1‰) in 18 out of 22 plants (Table S1; Figure S1a), suggesting that the sampled surface water was the main water source for *Rhizophora* trees in those estuaries. The observed ^2H -depletions of xylem water are therefore consistent with ^2H -discrimination during water uptake by roots in Pohnpeian estuaries.

Lin and Sternberg (1993) observed that $\delta^2\text{H}_{\text{XW}}$ values in *Avicennia germinans* were depleted by 1 - 2‰ relative to source water when grown in freshwater, but were depleted by up to 8‰ relative to source water when grown at a salinity of 28 ppt. Over the same salinity range, $\alpha^2_{\text{XW-SW}}$ in Pohnpeian *Rhizophora* trees decreased by 8‰ (Figure 4a), similar to that in the *A. germinans* measured by Lin and Sternberg (1993). In contrast to the *Rhizophora* from Pohnpei, Lin and Sternberg observed no difference in the magnitude of discrimination against ^2H by *Rhizophora mangle* from Florida growing in either freshwater or water with a salinity of 28 ppt.

Our results are consistent with the hypothesis that mangroves discriminate against ^2H during root water uptake, and that this discrimination increases as salinity increases. Increased ^2H -discrimination during water uptake could explain as much as 13‰ of the overall 30‰ decrease in both $\alpha_{\text{Tarax-SW}}$ and $\alpha_{\text{mC29-SW}}$ observed in *Rhizophora* spp. growing along estuaries in Pohnpei.

The situation is more complicated for *Rhizophora* growing around marine lakes in Palau, where the $\delta^{18}\text{O}_{\text{XW}}$ values were 1-3‰ lower than $\delta^{18}\text{O}_{\text{SW}}$ adjacent to the mangroves (Table S1; Figure S1b). This ^{18}O -depletion of xylem water suggests that the Palau mangroves had an additional water source. Potential alternative sources of water include subsurface water from the lakes, ground water, runoff during rain events, and foliar uptake of rain or dew.

It is highly unlikely that the sampled trees were using subsurface water, as this water was in all cases more saline than the surface water and had correspondingly enriched $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values (Smittenberg et al., 2011). Due to the shallow root structure of *Rhizophora* (Gill and Tomlinson, 1977; Hatchings and Saenger, 1987), they are typically confined to water sources within the upper meter of substrate (Lin and Sternberg, 1994). *R. mangle* in Florida has been observed using relatively fresh groundwater seeps from a depth of 1 m during the dry season (Ewe et al., 2007). Since we did not sample and analyze groundwater from Palau, we cannot rule out the possibility that these trees had access to a fresher groundwater source, with correspondingly lower $\delta^{18}\text{O}$ values.

Some work suggests that mangroves may opportunistically take up fresh water runoff during rain events (Wei et al., 2013; Reef and Lovelock, 2014; Santini et al., 2015). The light $\delta^{18}\text{O}_{\text{XW}}$ values from *Rhizophora* trees in Palau would be consistent with this strategy, since local meteoric freshwater had an average $\delta^{18}\text{O}$ value of -5‰ in Palau during the sampling period.

There is some evidence to suggest that mangroves may rely on foliar uptake of freshwater for a portion of their water (Hoste et al., 2011; de Groote, 2013; Reef and Lovelock, 2014). Foliar water uptake can be an important water source for plants in cloud forests (Johnson and Smith, 2008; Eller et al., 2013) and foggy coastal areas (Dawson, 1998; Burgess and Dawson, 2004). Reverse xylem flow, which would be consistent with foliar water uptake, has been

observed in mangroves at night (Rada et al., 1989; Hao et al., 2008; Hoste et al., 2011) and during rainfall events (Hoste et al., 2011). Foliar water uptake of rain or dew and corresponding reverse xylem flow would be also be consistent with relatively depleted $\delta^{18}\text{O}_{\text{XW}}$ values.

Measured $\delta^2\text{H}_{\text{XW}}$ values of Palau *Rhizophora* were less than would be expected based on their $\delta^{18}\text{O}_{\text{XW}}$ values and the local meteoric water line, which would predict $\delta^2\text{H}_{\text{XW}}$ values 6-14‰ higher than measured (Figure 6). This suggests that the measured $\delta^2\text{H}_{\text{XW}}$ values were offset from $\delta^2\text{H}_{\text{SW}}$ values not only because the trees were using alternative water sources, but also because they were discriminating against ^2H during water uptake. Because symplastic water uptake by roots in halophytes is reported to discriminate against ^2H by 2-11‰ (Lin and Sternberg, 1993; Ellsworth and Williams, 2007), the low $\delta^2\text{H}_{\text{XW}}$ values relative to surface water in *Rhizophora* mangroves is more consistent with water uptake by roots than with foliar uptake of water.

4.2. Leaf water enrichment in *Rhizophora*

Leaf water in *Rhizophora* was marginally enriched relative to xylem water across all sampled *Rhizophora* trees, and in 7 of 33 trees $\delta^2\text{H}_{\text{LW}}$ was slightly depleted relative to $\delta^2\text{H}_{\text{XW}}$ (Figures 2 and 4; Table S1). Measured $\delta^2\text{H}_{\text{LW}}$ values agreed well with the values predicted by the Péclet-Modified Craig-Gordon model of Kahmen et al. (2011) (Figure 3). The model predicted ~60% of the variability in $\delta^2\text{H}_{\text{LW}}$ values, despite sizable uncertainties in parameters such as humidity, temperature, stomatal conductance, path length, and water vapor $\delta^2\text{H}$ values.

The results of the leaf water model suggest that the relatively small ^2H -enrichment of leaf water relative to xylem water can be ascribed to relatively high ambient relative humidity, resulting in a small vapor pressure gradient across the leaf surface (Helliker and Ehrlinger, 2000; Farquhar et al., 2007). The increase in $\delta^2\text{H}_{\text{LW}}$ values with salinity depicted in Figure 2 can

primarily be attributed to the increase in $\delta^2\text{H}_{\text{XW}}$ values with salinity. The slopes of increase in both $\delta^2\text{H}_{\text{LW}}$ $\delta^2\text{H}_{\text{XW}}$ values with salinity are indistinguishable from each other, but shallower than that of $\delta^2\text{H}_{\text{SW}}$ values with salinity (Figure 2; Table 2). Increased ^2H fractionation during water uptake (Figures 4a and 4b) results in leaf water that is less enriched relative to surface water at high salinity than it would be if $\alpha^2_{\text{XW-SW}}$ was constant with salinity.

Because there was no correlation between salinity and $\alpha^2_{\text{LW-XW}}$ (Figures 4c and 4d), salinity-dependent changes in leaf water ^2H -enrichment relative to xylem water cannot account for the negative correlation between $\alpha_{\text{Lipid-SW}}$ and salinity (Figure 5). Therefore the third hypothesis proposed to explain the inverse relationship between salinity and $\alpha_{\text{Lipid-SW}}$ by Ladd and Sachs (2012), that increased salt residue on the leaf surface at higher salinity reduces the vapor pressure gradient across the leaf surface and therefore decreases leaf water enrichment relative to xylem water, cannot be correct. At least in humid environments such as Pohnpei and Palau, where the average relative humidity of 82% (www.nws.noaa.gov) is greater than the relative humidity maintained above saturated salt solutions (76%; O'Brien, 1984), alternate mechanisms must cause increased ^2H -discrimination in leaf lipids as salinity increases.

4.3. Relationship between salinity and biosynthetic ^2H fractionation in *Rhizophora* lipids

By measuring $\delta^2\text{H}_{\text{LW}}$ and $\delta^2\text{H}_{\text{Lipid}}$ values, the biosynthetic component of net fractionation, $\alpha_{\text{Lipid-LW}}$, can be calculated. With the exception of $n\text{C}_{29}$ -alkane from Pohnpei, there were not significant correlations between salinity and $\alpha_{\text{Lipid-LW}}$ (Table 2). These weak relationships may be due to the inherent noise in leaf water isotopes, which change significantly with meteorological conditions and the time of day (Kahmen et al, 2008; Ripullone et al., 2008). We attempted to avoid some of this temporal noise by modeling leaf water for climatological average conditions

at each site (Figure 3). Fractionation factors between modeled leaf water and all three lipids were not correlated with salinity in Palau (Table 2). In Pohnpei, the fractionation factors for both *n*-alkanes relative to modeled leaf water were significantly correlated with salinity (Table 2). The correlation for the taraxerol fractionation relative to modeled leaf water was not significantly correlated with salinity ($p = 0.09$; Table 2). However, the slope of the regression was not significantly different from those of the *n*-alkanes (Table 2), suggesting that there may be a similar relationship between salinity and biosynthetic fractionation for taraxerol. Overall, changes in biosynthetic fractionation can explain as much as two thirds of the 30‰ decrease in net fractionation for *Rhizophora* nC_{29} -alkanes in Pohnpei.

Variable biosynthetic fractionation has been observed in several other studies of hydrogen isotopes in lipids (e.g. Sessions, 2006; Zhang et al., 2009a; Feakins and Sessions, 2010; Romero and Feakins, 2011; Eley et al., 2013; Kahmen et al., 2013a). Variability in biosynthetic fractionation has been attributed to variable contributions of hydrogen from different NADPH sources (Schmidt et al., 2003; Zhang et al., 2009a; Dirghani & Pagani, 2013), the relative amounts of stored carbohydrates relative to recently photosynthesized carbohydrates used as molecular precursors (Yakir and DeNiro, 1990; Yakir, 1992; Roden and Ehrlinger, 2000; Sessions, 2006), and changes in the relative amounts of different metabolic products (for example, more compatible solutes produced by mangroves at high salinity) (Ladd and Sachs, 2012). The data collected and presented here does not definitively support or refute any of these possibilities, and future work should be focused on mechanistic studies of the causes of variable biosynthetic fractionation within and among plant species.

Changes in biosynthetic fractionation are difficult to determine when studying plants at a single point in time, as in this study. The measured leaf and xylem water and the climatology

used to calculate modeled values may not accurately reflect the water that was present in the leaf during the synthesis of the measured lipids. It is possible that the increased net fractionation observed in *Rhizophora* lipids at high salinity (Figure 5) was driven by variability in the timing of lipid synthesis. For example, if trees growing at high salinity selectively take up water during rain events (relatively low $\delta^2\text{H}$ values) and most of their lipids are synthesized at this time, they would have relatively depleted lipid $\delta^2\text{H}$ values relative to surface water compared to plants growing at low salinity that might be less influenced by the availability of rainwater. Alternatively, if high salinity plants conserve water by being more photosynthetically active during cooler times of day (early morning or evening), the leaf water used during photosynthesis might be expected to be less ^2H -enriched than leaf water at midday the samples were collected (Kahmen et al, 2008). Since the correlations between salinity and the apparent biosynthetic fractionation are relatively weak, variability in the relative timing of lipid synthesis could be at least part of the cause of observed changes in net fractionation in mangroves along a salinity gradient.

4.4 Relationship between salinity and net ^2H fractionation in *Rhizophora* lipids

On both Pohnpei and Palau, the net fractionation between surface water and taraxerol, $\alpha_{\text{Tarax-SW}}$, decreased with salinity (Figure 5). This is the first assessment of environmental controls on $\delta^2\text{H}_{\text{Tarax}}$, and demonstrates that $\delta^2\text{H}$ values of this *Rhizophora* biomarker (Killops and Frewin, 1994; Koch et al., 2003; Versteegh et al., 2004) have a very similar relationship to salinity as leaf wax *n*-alkanes (Ladd and Sachs, 2012). Likewise, $\alpha_{nC29-SW}$ from Pohnpei and $\alpha_{nC31-SW}$ from both Pohnpei and Palau were negatively correlated with salinity (Figure 5), suggesting that the negative correlation between salinity and $\alpha_{\text{Lipid-SW}}$ first observed in Brisbane

A. marina (Ladd and Sachs, 2012) may be more generally applicable to leaf lipids from mangroves.

The results of the present study demonstrate a relatively consistent negative relationship between salinity and for lipids synthesized in different biosynthetic pathways (acetogenic *n*-alkanes and the isoprenoid taraxerol) and in very different climates (humid subtropics in Brisbane and equatorial tropics in Micronesia and Palau). They also demonstrate a similar relationship between salinity and $\alpha_{\text{Lipid-SW}}$ in mangroves from two genera with different salt management strategies. Both *Rhizophora* and *Avicennia* exclude salt from entering their roots, but this filtration is more efficient in *Rhizophora*, which lacks *Avicennia*'s ability to secrete salt (Scholander et al., 1962).

Although the slope of the relationship between salinity and $\alpha_{\text{Lipid-SW}}$ was similar for the isoprenoid taraxerol and the acetogenic *n*-alkanes in Micronesian *Rhizophora*, $\delta^2\text{H}_{\text{Tarax}}$ values were depleted relative to $\delta^2\text{H}_{n\text{-alkanes}}$ in both Pohnpei and Palau (Figure 5; Table S1). Increased biosynthetic fractionation during the synthesis of isoprenoids relative to acetogenic lipids has been previously observed and attributed to a greater proportion of hydride derived from NADPH in the former (Sessions et al., 1999; Sessions, 2006; Zhang et al., 2009b).

Additionally, $\alpha_{\text{Lipid-SW}}$ was less sensitive to salinity in Micronesian *Rhizophora* than it was in *A. marina* growing in the Brisbane River (Ladd and Sachs, 2012). In Brisbane, a slope of $-1.5 \pm 0.3\text{‰ PSU}^{-1}$ was observed for both *nC*₃₁- and *nC*₃₃-alkanes from *A. marina*, while in this study the slopes of $\alpha_{\text{Lipid-SW}}$ for taraxerol and *n*-alkanes in *Rhizophora* spp. were between -0.7‰ to -1‰ ppt^{-1} (Figure 5; Table 2). The difference in sensitivity could be due to biological differences between the two types of mangroves, or to different environmental conditions.

It is possible that the lower relative humidity in Brisbane resulted in a stronger and steeper relationship between $\alpha_{\text{Lipid-SW}}$ and salinity than that observed in Micronesia. Saturated salt solutions maintain a relative humidity at their boundary layer of 76% (O'Brien, 1984). This is higher than the average relative humidity of 53% in Brisbane (<http://www.bom.gov.au>), but lower than the average relative humidity of 82% on both Pohnpei and Palau (www.nws.noaa.gov). Salt deposits on the leaf surface could therefore increase the relative humidity along the leaf surface in Brisbane, and reduce the vapor pressure differential (VPD) across the leaf surface. A lower VPD results in less leaf water enrichment (Flanagan et al., 1991; Ripullone et al., 2008), which would be expected to translate into less enriched leaf lipids.

One biological difference that could account for the different sensitivities of $\alpha_{\text{Lipid-SW}}$ to salinity in *Rhizophora* and *Avicennia* could be differing residence times of leaves on the two types of trees. In studies from Phuket, Thailand, and Darwin, Australia, *Rhizophora* leaves tended to persist longer on the tree, averaging 11.2 ± 0.2 months, as compared to *Avicennia* leaves that lasted on average for 6.3 ± 0.1 months (Wium-Andersen and Christensen, 1978; Coupland et al., 2005). These studies, both from continental regions with strong seasonality of precipitation, may not be representative of *Rhizophora* growing in regions with minimal seasonality, such as Pohnpei and Palau. Nevertheless, if this pattern does hold true for Micronesian mangroves, $\delta^2\text{H}_{\text{Lipid}}$ values from *Rhizophora* leaves might be more representative of long-term averages, while those in *Avicennia* may be more sensitive to seasonal conditions, and thereby more strongly influenced by salinity in the months just preceding sampling.

The potential for differences in the sensitivity of biologic fractionation to salinity among species highlights the importance of using a biomarker specific to an individual mangrove genus when reconstructing past salinity and water isotopes using the relationship observed here.

Taraxerol, which is highly specific to *Rhizophora* mangroves (Killops & Frewin, 1994; Koch et al., 2003; Versteegh et al., 2004), is a more suitable target for paleosalinity reconstructions than the more ubiquitous *n*-alkanes, which may be sourced from multiple types of mangroves, as well as from terrestrial plants.

5. CONCLUSIONS

The relationship between salinity and net $^2\text{H}/^1\text{H}$ fractionation in leaf *n*-alkanes and taraxerol was calibrated in *Rhizophora* mangroves from three estuaries and four brackish lakes on the Micronesian islands of Pohnpei and Palau. As salinity increased from 1-34 ppt, net fractionation between both lipid classes and surface water increased by ~30‰. For the *Rhizophora* biomarker taraxerol, the relationship between isotope fractionation and salinity was $\alpha_{\text{Tarax-SW}} = (-0.9 \pm 0.2\text{‰}) * S + 0.814 \pm 0.006$ ($R^2=0.29$; $p=0.0005$; $n=38$). Increased ^2H -discrimination during water uptake by roots can explain up to 43% of this signal.

Leaf water isotopes in the studied *Rhizophora* mangroves were marginally enriched relative to xylem water, irrespective of salinity. Measured enrichment of $\delta^2\text{H}_{\text{LW}}$ was broadly consistent with the predictions of a Péclet-Modified Craig-Gordon model of leaf water enrichment in terrestrial vascular plants that considers the effect of back diffusion in addition to ambient temperatures, humidity, stomatal conductance, xylem water $\delta^2\text{H}$, and water vapor $\delta^2\text{H}$. Therefore, mechanisms that invoke a change in leaf water enrichment relative to xylem water in response to salinity cannot explain the inverse relationship between salinity and net isotope fractionation in mangrove lipids. Instead, more plausible causes are changes in xylem water isotopes associated with opportunistic freshwater use, variable timing of leaf lipid production along a salinity gradient, or changes in $^2\text{H}/^1\text{H}$ fractionation during lipid biosynthesis as a direct

response to salt stress.

The observed 0.9‰ ppt^{-1} decrease *Rhizophora* leaf lipid $\delta^2\text{H}$ values supports the use of sedimentary taraxerol $\delta^2\text{H}$ values to reconstruct past changes in salinity. When combined with either $\delta^2\text{H}$ values of co-occurring algal lipids, or $\delta^{13}\text{C}$ values of taraxerol itself, both salinity and the $\delta^2\text{H}$ value of environmental water can be determined. Although the intercept of the linear least squares regression of $\alpha_{\text{tarax-SW}}$ with salinity is quite precise ($\pm 1\%$) the slope has an uncertainty of 22%. Estimates of salinity and water isotopes using the present field calibration will therefore be associated with moderately large uncertainties. Culturing mangroves at different salinities under controlled laboratory conditions ought to reduce those uncertainties.

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Table 1: Sites included in this study, with mean salinity, *Rhizophora* species present and surface water isotopes.

Site	Latitude (N)	Longitude (E)	Mean Salinity (PSS)	Species sampled	Mean water $\delta^2\text{H}$ (‰)	Mean water $\delta^{18}\text{O}$ (‰)
<i>Pohnpei, Federated States of Micronesia</i>						
Kamar 1	6°56.573'	158°13.591'	1 ± 5	<i>R. apiculata</i>	-24.3±1	-4.58±0.2
Kamar 2	6°57.276'	158°13.479'	4 ± 5	<i>R. apiculata</i>	-33.5±1	-4.13±0.2
Kamar 3	6°58.083'	158°13.317'	16 ± 5	<i>R. stylosa</i>	-12.0±1	-2.31±0.2
Kamar 4	6°58.849'	158°12.979'	23 ± 5	<i>R. stylosa</i>	-7.3±1	-1.58±0.2
Kamar 5	6°58.965'	158°13.054'	30 ± 5	<i>R. stylosa</i>	-6.7±1	-1.3±0.2
Sapwalap 1	6°53.204'	158°17.417'	2 ± 4.4	<i>R. apiculata</i>	-26.4±1	-5.12±0.02
Sapwalap 2	6°52.920'	158°17.768'	8 ± 4.4	<i>R. apiculata</i>	-21.4±1	-4.22±0.02
Sapwalap 3	6°52.935'	158°17.825'	10 ± 4.4	<i>R. apiculata</i>	-18.9±1	-3.77±0.02
Sapwalap 4	6°52.890'	158°18.109'	14 ± 4.4	<i>R. apiculata</i>	-16.5±1	-3.32±0.02
Sapwalap 5	6°52.733'	158°18.339'	19 ± 4.4	<i>R. apiculata</i>	-12.3±1	-2.42±0.02
Sapwalap 6	6°52.495'	158°18.536'	23 ± 4.4	<i>R. apiculata</i>	-9.0±1	-1.97±0.02
Sapwalap 7	6°52.448'	158°18.501'	25 ± 4.4	<i>R. apiculata, R. stylosa</i>	-6.6±1	-1.52±0.02
Sapwalap 8	6°52.232'	158°18.680'	29 ± 4.4	<i>R. stylosa</i>	-3.3±1	-0.92±0.02
Sapwalap 9	6°52.252'	158°18.604'	31 ± 4.4	<i>R. apiculata, R. mucronata</i>	-2.4±1	-0.77±0.02
Sapwalap 10	6°51.742'	158°19.475'	34 ± 4.4	<i>R. stylosa</i>	0.1±1	-0.77±0.02
Sapwalap 11	6°52.123'	158°20.313'	34 ± 4.4	<i>R. stylosa</i>	0.1±1	-0.32±0.02
Soundau 1	6°54.712'	158°08.952'	4 ± 2.8	<i>R. apiculata</i>	-23.1±1	-0.32±0.02
Soundau 2	6°54.507'	158°07.654'	14 ± 2.8	<i>R. apiculata</i>	-15.4±1	-4.39±0.13
Soundau 3	6°54.403'	158°07.517'	18 ± 2.8	<i>R. apiculata, R. mucronata</i>	-12.4±1	-3.03±0.13
Soundau 4	6°54.343'	158°07.332'	22 ± 2.8	<i>R. mucronata</i>	-9.3±1	-2.49±0.13
Soundau 5	6°54.187'	158°07.197'	26 ± 2.8	<i>R. stylosa</i>	-6.2±1	-1.94±0.13
Soundau 6	6°54.064'	158°06.945'	30 ± 2.8	<i>R. stylosa</i>	-3.1±1	-1.4±0.13
Soundau 7	6°54.135'	158°06.758'	33 ± 2.8	<i>R. stylosa</i>	-0.8±1	-0.86±0.13
<i>Palau</i>						
Little Croc Lake	7°09.04'	134°21.33'	8 ± 2.2	<i>R. apiculata</i>	-29.4±1	-4.43±0.15
Big Croc Lake	7°09.34'	134°22.12'	20 ± 3.2	<i>R. mucronata</i>	-12.5±1	-1.48±0.11
Long Lake 1	7°14.908'	134°24.527'	32 ± 1.1	<i>R. mucronata</i>	-4.64±1	-0.39±0.03
Long Lake 2	7°14.908'	134°24.527'	32 ± 1.1	<i>R. mucronata</i>	-4.64±1	-0.5±0.05
Lagoon 1	7°20.123'	134°29.834'	29 ± 1	<i>R. mucronata</i>	1.8±1	-0.16±0.06
Lagoon 2	7°20.80'	134°27.31'	33 ± 1	<i>R. mucronata</i>	1.8±1	0.7±0.04
Lagoon 3	7°20.826'	134°27.295'	32 ± 1	<i>R. apiculata</i>	1.8±1	0.56±0.02

Table 2

	<i>Pohnpei</i>					<i>Palau</i>				
Variable	Slope	y-intercept	R ²	p	n	Slope	y-intercept	R ²	p	n
Surface water δ ² H (VSMOW, ‰)	0.81 ± 0.02	-27.6 ± 0.5	0.96	< 0.0001	50	1.14 ± 0.03	-36.9 ± 0.9	0.98	< 0.0001	26
Surface water δ ¹⁸ O (VSMOW, ‰)	0.142 ± 0.003	-5.16 ± 0.007	0.98	< 0.0001	50	1.197 ± 0.008	-6.1 ± 0.2	0.96	< 0.0001	26
Xylem water δ ² H (VSMOW, ‰)	0.56 ± 0.06	-32 ± 1	0.81	< 0.0001	22	0.9 ± 0.1	-52 ± 3	0.88	< 0.0001	12
Xylem water δ ¹⁸ O (VSMOW, ‰)	0.10 ± 0.01	-4.6 ± 0.2	0.81	< 0.0001	22	0.14 ± 0.02	-6.6 ± 0.4	0.87	< 0.0001	12
Leaf water δ ² H (VSMOW, ‰)	0.4 ± 0.1	-24 ± 3	0.38	0.0028	21	0.3 ± 0.3	-32 ± 8	0.09	0.34	12
Leaf water δ ¹⁸ O (VSMOW, ‰)	0.11 ± 0.03	-1.3 ± 0.9	0.36	0.0037	21	0.05 ± 0.09	-2 ± 2	0.03	0.57	12
α ² _{XW-SW}	-0.00030 ± 9*10⁻⁵	0.996 ± 0.002	0.34	0.0043	22	-0.0002 ± 0.0002	0.985 ± 0.004	0.17	0.19	12
α ¹⁸ _{XW-SW}	-4*10⁻⁵ ± 1*10⁻⁵	1.0000 ± 0.0002	0.34	0.0045	22	4*10 ⁻⁵ ± 2*10 ⁻⁵	0.9991 ± 0.0006	0.22	0.13	12
α ² _{LW-XW}	-0.0001 ± 0.0001	1.008 ± 0.003	0.06	0.28	21	-0.0006 ± 0.0004	1.021 ± 0.009	0.24	0.11	12
α ¹⁸ _{LW-XW}	4*10 ⁻⁵ ± 5*10 ⁻⁵	1.002 ± 0.001	0.03	0.46	21	-3*10 ⁻⁵ ± 0.0001	1.004 ± 0.003	0.01	0.83	12
α _{Tx-LW} (measured)	-0.0007 ± 0.0004	0.816 ± 0.009	0.14	0.097	21	-4.57*10 ⁻⁵ ± 0.0004	0.80 ± 0.01	0.00	0.91	12
α _{Tx-LW} (modeled)	-0.0006 ± 0.0003	0.809 ± 0.008	0.14	0.099	21	-9*10 ⁻⁵ ± 0.0003	0.801 ± 0.008	0.01	0.76	12
α _{C29-LW} (measured)	-0.0008 ± 0.0002	0.874 ± 0.006	0.54	0.0068	12	0.0002 ± 0.0008	0.85 ± 0.02	0.01	0.83	7
α _{C29-LW} (modeled)	-0.0006 ± 0.0002	0.865 ± 0.006	0.45	0.017	12	0.0002 ± 0.0006	0.85 ± 0.01	0.01	0.80	7
α _{C31-LW} (measured)	-0.0005 ± 0.0003	0.869 ± 0.007	0.23	0.071	15*	0.0001 ± 0.0005	0.85 ± 0.01	0.01	0.81	16
α _{C31-LW} (modeled)	-0.0005 ± 0.0002	0.863 ± 0.004	0.31	0.030	15*	-0.0002 ± 0.0002	5*10 ⁻⁵ ± 0.0003	0.00	0.89	12
α _{Tx-SW}	-0.0010 ± 0.0003	0.817 ± 0.007	0.27	0.0068	26	-0.0007 ± 0.0003	0.806 ± 0.007	0.38	0.034	12
α _{C29-SW}	-0.0010 ± 0.0003	0.874 ± 0.008	0.44	0.010	14	-0.0006 ± 0.0004	0.85 ± 0.01	0.28	0.22	7
α _{C31-SW}	-0.0007 ± 0.0002	0.871 ± 0.005	0.42	0.0037	18*	-0.0007 ± 0.0003	0.858 ± 0.007	0.37	0.037	12

Table 2 Summary of linear regression statistics for all water isotopes and various fractionation factors with salinity. Bolded relationships are significant at the $p < 0.05$ level. One outlier was removed from the Pohnpei nC_{31} -alkane analyses. This sample was from a tree where most of the leaf had a strange moss growing on their surface was not present at any other sites. Although the sampled leaves did not have this moss present at the time of sampling, they had virtually no nC_{29} -alkanes, anomalously low nC_{31} -alkane concentrations, and anomalously low nC_{31} -alkane δ^2H values.

Figure Captions:

Figure 1: Map of field sites in Palau (left panel) and Pohnpei (right panel). Stars indicate locations of lakes sampled on Palau and estuaries sampled on Pohnpei. Images of Palau and Pohnpei are from Google Earth.

Figure 2: Surface (blue), xylem (brown) and leaf (green) water isotopes as a function of salinity. Panel a shows $\delta^2\text{H}$ values from Pohnpei estuaries and panel b shows $\delta^2\text{H}$ values from Palau lakes. Panels c and d contain the corresponding $\delta^{18}\text{O}$ values. The x-error bars for Pohnpei (panels a and c) represent the standard error in the regression between salinity and distance from the river mouth. The x-error bars from Palau represent the standard deviation of historical salinity measurements at the site over the previous six years. Y-error bars represent the standard deviation of triplicate water isotope measurements. The slopes and intercepts of all regression lines shown here, as well as R^2 values and p values are listed in Table 2.

Figure 3: Relationship between modeled and measured leaf water $\delta^2\text{H}$ values for *Rhizophora* spp. in Pohnpei and Palau. Modeled leaf water values are from a Peclet-modified Craig-Gordon model of leaf water enrichment. Error bars on modeled values (x-axis) are 1σ uncertainties determined from a 1000 iteration Monte Carlo simulation that included all uncertainties associated with individual parameters or measurements. Error bars of measured $\delta^2\text{H}$ values (y-axis) are 1 standard deviation of triplicate measurements.

Figure 4: Relationship between salinity and hydrogen fractionation between different water pools. Panels a and b show ^2H fractionation between xylem and surface water ($\alpha^2_{\text{xw-sw}}$) in (a) Pohnpei estuaries and (b) Palau lakes. Panels c and d show ^2H fractionation between leaf and xylem water ($\alpha^2_{\text{LW-XW}}$) in (a) Pohnpei estuaries and (b) Palau lakes. Salinity error bars are as described in Figure 2 and section 2.1. Error bars for fractionation factors are propagated 1σ uncertainties from triplicate measurements of each water isotope measured to calculate the fractionation factors.

Figure 5: Relationship between salinity and net fractionation in mangrove leaf lipids ($\alpha_{\text{Lipid-SW}}$) for (a) Pohnpei taraxerol, (b) Palau taraxerol, (c) Pohnpei $n\text{C}_{29}$ -alkanes, (d) Palau $n\text{C}_{31}$ -alkanes, (e) Pohnpei $n\text{C}_{31}$ -alkanes, (f) Palau $n\text{C}_{31}$ -alkanes. Salinity error bars are as described in Figure 2 and section 2.1. Error bars for fractionation factors are propagated 1σ uncertainties from triplicate measurements of surface water isotopes and lipid isotopes. One outlier from the Pohnpei $n\text{C}_{31}$ -alkane sample set is highlighted in red and was not included in the regression.

Figure 6: Measured xylem water $\delta^2\text{H}$ values from Palau *Rhizophora* plotted relative to those predicted by measured xylem water $\delta^{18}\text{O}$ values and the relationship between the two isotopes in surface water (LWL = Local Water Line). Error bars on measured values are 1 standard deviation of triplicate measurements.

Figure 1

Figure 2

Pohnpei *Palau*

Figure 3

Figure 4

Pohnpei *Palau*

Figure 6

Predicted $\delta^2\text{H}_{\text{xw}}$ (VSMOW, ‰)