Decomposition of mangrove litter under experimental nutrient loading in a fringe *Rhizophora mangle* (L.) forest

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ABSTRACT

Carbon (C) cycling is an important attribute of mangrove forests that relates to the structure, function, and resilience of mangroves under environmental change. Increased nutrient enrichment in tropical coastal waters may influence C cycling through organic C mineralization. For example, by alleviating nutrient limitation of the heterotrophic microbial community,

nutrient enrichment may enhance C mineralization and facilitate a loss of within-stand C sequestration. Here, we enriched a coastal fringe *Rhizophora mangle* (L.) mangrove system for two years with two fertilizer regimes to mimic agriculture runoff ("+high" N:P ratio of 50:1) and urban runoff ("+moderate" N:P ratio of 16:1) scenarios as follows: (1) annual loading rate of 70 g N m^{-2} $year^{-1}$ and 3.1 g P m^{-2} $year^{-1}$ or (2) annual loading rate of 70 g N m^{-2} $year^{-1}$ and 9.7 g P m^{-2} $year^{-1}$. C mineralization was measured as microbial respiration rates from the forest floor and litter decomposition rates. While decomposing leaf litter and green leaves had lower molar C:N under the +moderate N:P fertilization course, neither fertilization scenario produced an effect on C mineralization processes compared with ambient conditions. Substrate CO_2 flux rates were not different among treatments and ranged from 1.15 to 1.81 µmol CO_2 m^{-2} s^{-1} (3.0 to 4.8 g CO_2 m^{-2} day^{-1}) following 72 weeks of fertilization and 0.58 to 1.55 µmol CO_2 m^{-2} s^{-1} (1.5 to 4.1 g CO_2 m^{-2} day^{-1}) 30 weeks following the end of the experiment. Time to 50% decay of above-ground leaf litter ranged from 61 to 110 days (average 79 days). Below-ground leaf litter material was fully decomposed by 22 months after burial. A ^{15}N pulse-recovery suggests that the majority of the retained fertilizer (22.2 $\pm$ 4.4% at 10 months following spike) was taken up by fine roots, though this did not significantly affect CO_2 flux from the forest floor. This work demonstrates that nutrient enrichment by aqueous delivery does not strongly affect organic carbon mineralization in a coastal fringe mangrove within two years. Environmental conditions, substrate quality, and location may play a more substantial role in mangrove C dynamics compared with short-term aqueous-based nutrient enrichment.

1. Introduction

Mangrove ecosystems are often attributed for providing services such as storm buffering (Das and Vincent, 2009), wastewater depuration (Corredor and Morell, 1994), sea level rise resilience (McKee et al., 2007), enhancement of secondary production (Mumby et al., 2004), and carbon (C) sequestration (Bouillon et al., 2008; Donato et al., 2011). Each of these processes is directly linked to organic matter production and turnover rates within the mangrove system; thus the mechanisms and driving factors of organic matter production, decomposition, and retention are necessary elements for understanding mangrove ecosystem services. The mangrove habitat – ranging from warm temperate to tropical coastlines – is subject to increasing anthropogenic nutrient (nitrogen, N and phosphorus, P) loading rates due to human population growth, urban development, and changing land use practices (Corredor et al., 1999; Downing et al., 1999; Nixon et al., 2007). Globally, regions with the greatest increase of inorganic nutrient input to coastal waters (over 500 kg of dissolved inorganic nitrogen (DIN) km^{-2} $year^{-1}$) include densely populated areas of the Indian Ocean, southwest Pacific, and to a smaller extent the Caribbean Sea (Deegan et al., 2012) where mangroves dominate the vegetated coastline. While temperate coastal systems are primarily N-limited (Howarth and Marino, 2006), tropical coastal ecosystems have been found to be either N and/or P limited depending on factors such as sediment delivery, N-fixing microbial activity, and sediment cation exchange capacity (Corredor et al., 1999; Tappin 2002; Reef et al., 2010). The rapid anthropogenic enhancement of biologically-available inorganic nutrients (Howarth, 2008) combined with the central importance of the C cycle for coastal ecosystem function drives the need for an understanding of organic C processes under enriched nutrient conditions in mangrove systems.

Within the mangrove forest, the main source of organic material comes from tree production (Bouillon et al., 2008; Kristensen et al., 2008). The evolutionary effects of resource limitation and environmental regulation (*sensu* Twilley and Rivera-Monroy, 2005) are physiological adaptations by the mangrove trees to conserve nutrients, limit herbivory, and maintain internal osmotic balance (Ball, 1988). These adaptations lead to low energetic quality of mangrove organic matter (OM): Nutrient (nitrogen, N and phosphorus, P) concentrations of senescent leaves are low due to efficient resorption before leaf abscission (Feller et al., 1999); high tannin levels deter herbivory of live leaves (Feller, 1995); high leaf sclerophylly limits water loss from evapotranspiration (Ball, 1988). Given the poor nutritional quality of this refractory material, higher trophic levels (e.g., shredder crabs) do gain the majority of their energy from the direct assimilation of mangrove OM (Méziane et al. 2006). However the detrital pathway as described by Odum and Heald (1975) postulates that mangrove OM quality is enhanced and made available for secondary trophic level use via microbial decomposition and fungal colonization, which in turn are the main sources of energy for resident shredding consumers (Méziane et al. 2006). Coincident with this pathway, microbial OM remineralization promotes nutrient recycling within the forest system for primary producer uptake (Holguin et al., 2001). Thus the rate of mangrove OM degradation is a bottleneck step for system-wide energy flow, with slow rates supporting within-stand nutrient conservation (Reef et al., 2010). Furthermore, any mangrove OM that is not directly consumed, decomposed, or physically exported becomes retained within the soil matrix (Cebrián et al., 1998). Mangrove systems with low decomposition rates and low flushing conditions develop deep structural platforms of peat and a biological capacity to maintain elevation relative to sea level rise (McKee et al., 2007). An

104 enhancement of OM decomposition in coastal wetlands by nutrient enrichment may deter this
105 ability to maintain structural peat (Deegan et al., 2012).

106 Increased nutrient availability within a system may directly or indirectly affect OM
107 decomposition rates. Indirect effects of nutrient enrichment include changes to the substrate
108 quality or input rate. Nutrient resorption before leaf senescence and abscission can be regulated
109 by the local availability of the same nutrient (Vitousek,1984; Schlesinger et al., 1989;
110 Killingbeck,1996). Feller et al. (1999; 2009) demonstrated a negative relationship between
111 nutrient input and leaf nutrient resorption in fertilized mangrove systems, allowing more of the
112 nutrient to be "lost" to the benthic community under enriched conditions. In addition, Feller
113 (1996) demonstrated that leaf sclerophylly decreased by 20-60% with phosphorus enrichment in
114 severely P-limited dwarf mangroves. With regards to organic material input rates, results are
115 variable based on site-specific conditions. Boto and Wellington (1983) measured greater leaf
116 production within the first year of fertilization of a north Queensland mangrove, presumably
117 enhancing the amount of organic matter available to the decomposer community. N limitation
118 relief also enhanced above-ground production within fringe and dwarf mangrove stands in
119 Florida (Feller et al. 2003; Whigham et al. 2009). However Tam et al. (1998) reported no change
120 in the amount of mangrove litter production with fertilization from wastewater discharge.
121 Depending on the nutrient limitation status of the mangrove forest, it is reasonable to predict
122 greater rates of OM decomposition given a more favorable quality and availability of substrate
123 under enriched nutrient conditions.

124 An example of direct effect to OM decomposition by nutrient enrichment would be
125 nutrient limitation relief for the microbial decomposer community by supplementing inorganic
126 nutrients in place of organic substrate-derived nutrients (Debusk and Reddy, 2005). Castañeda-

Moya et al. (2011) measured higher rates fine-root turnover in a mangrove system relieved of P limitation compared with a P-limited system. Similarly, Feller et al. (1999) found higher rates of decay for buried cotton strips when fertilized with P. In contrast, Poret et al. (2007) found that nutrient availability was not a main driver of below-ground decomposition rates.

Keuskamp et al. (2017) shed light upon the complicated discrepancy of nutrient limitation by producers vs microbial decomposers: Whereas mangrove OM input was limited by P, microbial decomposers were N-limited due to the protein-binding tannins from the leaf litter. While nutrient relief may enhance microbial decomposer activity, there is evidence of negative feedback by nutrient enrichment if the inorganic nutrient supply decreases the "need" for OM breakdown to collect nutrients (Knorr et al., 2005; Craine et al., 2007). The overall effect of nutrient enrichment on OM decomposition continues to present as complex and variable and requires additional evidence from a variety of locations.

Previous studies have demonstrated enhanced heterotrophic microbial activity under direct nutrient stimulus in freshwater systems (Robinson and Gessner 1999), salt marshes (Wigand et al., 2009; Deegan et al., 2012), and subtropical wetlands (Corstanje et al., 2006; Debusk and Reddy 2005). Mangrove studies, on the other hand, have presented mixed results of OM decomposition under enriched nutrient conditions. Tam et al. (1998) found no relationship of mangrove litter decay rates along a nutrient gradient extending from a sewage treatment plant at Shenzhen, China. Similarly, Feller et al. (1999) found no difference in decomposition rates of submerged leaves within a fertilized dwarf Rhizophora mangle at Twin Cays, Belize, although below-ground decomposition was enhanced with buried fertilizer cores. Keuskamp et al. (2015) used a multi-factorial design to differentiate source material enrichment versus direct nutrient enrichment of decomposing litter at sites in Belize and Florida. In a P-limited mangrove system,

150 enhancement of litter quality with P fertilization stimulated OM decomposition rates; however,
151 direct fertilization of the decomposition matrix without pre-enriched litter did not affect OM
152 decomposition rates. From these works it appears that OM decomposition and retention are
153 modulated via the indirect pathway of substrate quality and quantity and not necessarily by direct
154 nutrient input.

155 To date, the majority of mangrove nutrient enrichment field studies have been conducted
156 using buried fertilizer (Boto and Wellington 1983; Feller 1995; Feller et al. 2003; Lovelock et al.
157 2009; Simpson et al. 2020). The benefit of this method is that fertilizer is not lost by tidal action
158 or volatilization, but a limitation is that the added nutrients are not first encountered and
159 modified by the microbial community or biogeochemical context on the surface of the forest
160 floor (Whigham et al. 2009). Anthropogenic nutrients are often transported to coastal
161 ecosystems via water channels (Howarth et al. 1996) and are likely to interact with components
162 of the intertidal system differently compared with belowground cores of fertilizer (Deegan et al.
163 2007). Therefore, this study will deliver nutrients by surface-level aqueous application to more
164 closely mimic current-day anthropogenic nutrient input.

165 This study examines organic C mineralization under direct nutrient application as well as
166 sourced organic material from mangrove trees that have encountered nutrient enrichment. The
167 main purpose of this study is to report on the response by fast-turnover (e.g., microbial)
168 communities within a two-year period of periodic nutrient enrichment with three scenarios of
169 nutrient enrichment of a fringe mangrove (Lugo and Snedaker 1974). The fertilizer treatments
170 were +high N:P (50N:1P), +moderate N:P (16N:1P), and control (ambient channel water).
171 +High N:P fertilizer represents nutrient input from agriculture sources (sugarcane watershed
172 drainage N:P = 43.9; Faithful et al. 2007), and +moderate N:P fertilizer represents nutrient input

173 from urban sources (urban drainage N:P = 12.6; Hopkinson and Day 1980). The rate of N
174 addition is equivalent for both fertilization scenarios, with P addition altered for the appropriate
175 ratio. Fertilizer was applied on a plot-level (4 m^2) scale every-other week to mimic pulsing
176 events by aqueous flow. Microbial activity was measured via above- and below-ground litter
177 decomposition and CO_2 flux from the forest floor to make inferences about the effect of aqueous
178 pulsing of nutrients over two years.

179 **2. Methods**

180 *2.1. Study system*

181 This study was conducted in a fringe mangrove (Lugo and Snedaker 1974) at the eastern
182 side of the mouth of Jobos Bay (17° 55.5'N 60° 12.2'W) in southeastern Puerto Rico (Figure 1).
183 The vegetation community is dominated by *Rhizophora mangle* (L.) with intermittent
184 *Laguncularia racemosa* (L.) C.F. Gaertn, which extends from the shoreline 10 to 20 m and
185 transitions into an *Avicennia germinans* (L.) forest. Mean annual rainfall ranges 106 – 114 cm,
186 with a dry season from December through March and a rainy period from July through October
187 (Capella, 2008). Air temperature is relatively constant throughout the year, with a range of
188 average daily temperature from 16.5 – 35.7° C and an annual average of 26.4° C. Several small
189 rivers and intermittent streams empty into the bay, but no freshwater discharge reaches the
190 mangrove cays at the mouth of the bay. The tidal profile is mixed diurnal microtidal (17 – 36 cm
191 tidal range) with largest tides of the year occurring in October (Field 2008).

192 Sites were selected to reflect the least amount of relative anthropogenic nutrient input,
193 based on the following information: (i) Mangroves at the mouth of Jobos Bay receive
194 overflowing tidal water from a channel that flows directly from the Caribbean Sea into the bay;
195 (ii) Stable isotope (^{15}N) signatures suggest that rooted vegetation communities of Jobos Bay are

196 disconnected from mainland-derived nutrient runoff (Bowen and Valiela, 2008); (iii) Any
197 potential input from the nearest residential community (Punta Pozuelo) would be buffered by a
198 region of sand dunes and forest, and sewage from the community is discharged away from the
199 bay; (iv) The closest Jobos Bay National Estuarine Research Reserve monitoring station has
200 consistently lower ambient nutrient (DIN, DIP) levels compared with stations within the bay
201 (NERRS 2015).

202 *2.2. Nutrient enrichment*

203 A two-year nutrient enrichment protocol (October, 2011 to September, 2013) was
204 conducted with three levels of nutrient enrichment: +high N:P (50N:1P), +moderate N:P
205 (16N:1P), and control (ambient channel water). The total amount of N added was equivalent for
206 both enriched treatments, with different amounts of added P. +High N:P fertilization represented
207 nutrient input from agriculture sources (sugarcane watershed drainage N:P = 43.9; Faithful et al.,
208 2007), and +moderate N:P fertilization represented nutrient input from urban sources (urban
209 drainage N:P = 12.6; Hopkinson and Day, 1980). The annual loading rates were 70 g N m^{-2} $year^{-1}$
210 and 3.1 g P m^{-2} $year^{-1}$ ("agriculture" +high N:P fertilizer), 70 g N m^{-2} $year^{-1}$ and 9.7 g P m^{-2}
211 $year^{-1}$ ("urban" +moderate N:P fertilizer), or 1 L local channel water without fertilizer
212 ("ambient"). With the assumption that the experimental area is submerged by 6 cm of tidal
213 water for half of the year (approximate mean high water), the total annual nutrient loading is
214 calculated to be approximately 30 times the amount of DIN loading by tidal overflow (NERRS,
215 2015). An estimate of P loading increase is difficult to make because channel water phosphate
216 levels were below the detection limit over 80% of the experiment time period (NERRS, 2015).

217 The experimental design was a randomized block, where three blocks were spaced at
218 least 100 m apart along the coastal edge (5 m from the shoreline). Within each block, three 4 m^2

quadrats were established with at least 3.5 m spacing to prevent nutrient exchange among quadrats. A qualitative flow test using dye and particulate material was conducted to determine that lateral flow was not occurring among quadrats during a tidal cycle. Nutrients were added every other week, and most often coincided with low tide to ensure that nutrients diffused into the soil. For each quadrat, a stock solution of NH_4Cl and KH_2PO_4 was prepared in 250 mL deionized water at the laboratory within one week of application, which was then mixed with 1 L of local channel water immediately before application with a hand-pumped sprayer. Care was taken to keep the spray nozzle close to the forest floor to minimize volatilization, and to spray evenly throughout the quadrat. Workers stepped outside of the quadrat perimeter or balanced on sturdy prop roots so the forest floor was not disturbed.

To determine whether fertilizer stayed within the enriched quadrats, a one-time spike of ^{15}N-ammonium sulfate (95.2% isotope enrichment) was applied over two concurrent days in August 2013 (21 months after the start of the experiment) to the +moderate N:P quadrats. Tidal inundation of the plots occurred following each application. The ^{15}N-enriched application followed the fertilization procedure described above, with designated hand sprayers to prevent subsequent isotopic contamination. Within the first month following the spike, 1.4 to 4.6% was calculated to be retained in the standing litter and 5.2% in the top sediment layer. Fine roots were not sampled during this initial period. At 10 months following the spike, 16.6 to 23% of the ^{15}N tracer was recovered within all sampled biomass components (leaves, surface sediment, decomposing litter, and fine roots) with over 50% of the ^{15}N tracer recovery in fine roots. Larger roots, wood, and deep cores were not sampled; therefore this may be an underestimate of the total fertilizer retention (Jessen, 2016).

241 Limited resources prevented a full sampling of all treatment quadrats for C:N analyses
242 (described below). We report on characterization of quadrats receiving ambient treatment
243 compared with +moderate N:P treatment, which is the highest amount of total nutrient addition.
244 As the +high N:P treatment delivers the same level of N addition but lower amounts of P, we
245 made the assumption that +moderate N:P treatment conditions would present the greatest
246 opportunity to detect change.

247 *2.3. Sediment characterization*

248 At the end of the experiment, surface layer (2 to 5 cm) sediment cores were sampled to
249 determine bulk density, percent organic matter content, and total C and N. For bulk density, two
250 small 3 cm-diameter cores were sampled to a depth of 4.5 cm with a volumetric plastic corer
251 (30.4 cm^3), after fine roots were cut from around the corer during insertion to avoid compaction.
252 The samples were dried at 45° C until a constant weight was reached. The same samples were
253 used to calculate percent organic matter content by burning at 550° C for at least six hours to
254 obtain an ash-free dry weight.

255 The organic layer of sediment (0 to 2 cm) was sampled with a cut-off syringe (2 cm
256 diameter) at three locations within each quadrat. The samples were combined for processing and
257 analysis. Mangrove roots with a diameter greater than 2 mm were removed, and the
258 sediment/fine root samples were ground with a mortar and pestle. Triplicate subsamples of the
259 homogenized sediment were analyzed for total C and N on a Thermo Scientific Flash EA-112
260 CHNSO analyzer (U. S. EPA Atlantic Coastal Environmental Sciences Division, Narragansett,
261 RI) and averaged for the reported C:N value.

262 *2.4. Field measurements*

263 *2.4.1. CO_2 flux measurements*

264 Carbon dioxide (CO_2) flux from the forest floor under unflooded conditions was
265 measured during the dry season (March 2013; 72 weeks from the start of nutrient enrichment).
266 All three experimental blocks were measured during the day in March 2013. A comparative
267 measurement was made for all blocks during daytime in May, 2014, following 30 weeks of
268 "recovery" from chronic nutrient enrichment pulsing. Each sampling period was conducted over
269 a course of three days (one block per day).

270 For each measurement of CO_2 flux, three to five replicate locations within each quadrat
271 (at least nine replicates per treatment) were haphazardly selected to place sediment collars. The
272 selection criteria were areas that did not have significant (> 5 cm^2) macroalgae cover, *L.*
273 *racemosa* pneumatophores or *R. mangle* aerial roots, numerous (> 5) or large (> 1 cm) crab
274 burrows, and had not been previously disturbed by coring. If a large below-ground root blocked
275 a complete collar insertion, a new location was selected. To maintain consistency, any large
276 pieces of recent litter fall (e.g., new leaf fall that had not become part of the sediment matrix)
277 were carefully removed by hand and the area was left to rest for at least 30 minutes after collar
278 insertion. PVC sediment collars (6 cm height, 10 cm diameter) were hand-pushed or gently
279 hammered into the ground to at least 4 cm depth. The exposed collar height was measured at
280 four points and the average value was used to calculate the final chamber volume (approximately
281 995.4 cm^3 total volume). Pertinent features of the measurement area (crab burrow count and
282 size, macro-algae area) were measured. A soil temperature probe was inserted 4 cm into the soil
283 approximately 1 cm from the outer edge of the collar at the time of each measurement.

284 A Li-Cor 8100 Infrared Gas Analyzer (IRGA) chamber was placed over each soil collar,
285 using foam rings to ensure an air-tight seal. Ambient CO_2 concentration within the chamber was
286 measured every two seconds for a total incubation of three and a half minutes. The initial 30-
287 seconds of the incubation period was not included in this analysis to allow for gas flushing
288 through the lines.
289 The values within an incubation period were plotted using the software package LI-8100
290 Viewer. A linear regression was performed to calculate a rate of change in CO_2 concentration
291 over the incubation period. The coefficient of variation (flux CV) for each regression ranged
292 from 1.07 to 1.71. The CO_2 concentration units ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$), were calculated using the
293 assumption that at 25°C, 1 atm pressure and a molar mass of 12.0 g C mol^{-1} CO_2, the molar
294 volume was 24.5 L mol^{-1}. Flux values calculated for each incubation (replicate) were averaged
295 by quadrat prior to statistical analysis.
296 *2.4.2. Above-ground Litter Decomposition*
297 Decomposition of leaf material on the forest floor was measured with litter
298 decomposition bags (Karberg et al., 2008) over a two-month period from July to October (2013).
299 Locally-sourced senescent leaves that had recently fallen on the forest floor (identified as yellow
300 to light-gold color) were collected within each experimental quadrat and kept separate according
301 to the treatment and block. It was assumed that leaves collected within each quadrat were
302 sourced by trees that encountered nutrient treatment for that quadrat. The leaves were kept cool
303 during transport to the laboratory, then rinsed in distilled water and air-dried for 24 hours at
304 room temperature. Four to five air-dried leaves were sealed within a 14 cm x 14 cm fiberglass
305 mesh litter bag with 1 mm^2 pores to exclude macroinvertebrate shredders. Care was taken to

306 ensure that the leaves lay flat and separated within the litter bags for consistent exposure to the
307 forest surface.
308 Approximately 50 additional leaves were collected from the surrounding area of all
309 experimental units for a fresh weight to dry weight conversion factor. The leaves were rinsed
310 and air-dried for 24 hours, weighed ("fresh weight"), then dried at 45° C until a constant weight
311 was reached ("dry weight"). A linear regression of fresh weight to dry weight was used to
312 convert the fresh weight of the initial litterbag samples to dry weight for comparison with the
313 oven-dry recovered leaf tissue.
314 Immediately after preparation, five bags were deployed at each experimental quadrat (day
315 0). One litterbag was removed from each quadrat at 9, 32, 60, and 90 days and placed in a cooler
316 for transport to the laboratory. At the laboratory, the contents of the bag were submerged in
317 distilled water and gently cleaned to remove sediment. After cleaning, the contents of each bag
318 were dried at 45°C until a constant weight was reached.
319 The amount of organic material lost over the decomposition period was expressed as a
320 percentage of the initial mass:
321
322 *Percent mass remaining* = [(final dry weight)/(calculated initial dry weight)]*100
323 *(Eq. 1)*
324
325 Most of the decomposing litter displayed a linear decay trend within the study time;
326 therefore, a linear regression with Microsoft Excel was used to compare the mass loss among all
327 of the decomposition time series. Based on similar mangrove litter decomposition studies (Tam
328 et al., 1998: 10 weeks; Ake-Castillo et al., 2006: 21.5 weeks; Keuskamp et al., 2015: 36 weeks),

329 the degradation trends would likely follow an exponential decay if the incubation period were
330 longer. To compare our decay rates (k) with other studies that used an exponential decay model,
331 we used the linear model to calculate the time to reach 50% decay ($t_{0.5}$), which was then fit
332 within the experimental decay model $X = X_0 e^{-kt}$,where X is the final sample weight, X_0 is the
333 initial sample weight, k is the decay rate, and t is the time of decomposition in days. Thus k is
334 calculated by
335
336 $$[ln\ (0.5)] / t_{0.5} = k \qquad (Eq.\ 2)$$
337
338 Collected litter samples were ground using a Wiley-grinding mill with a #20 sieve. To
339 determine isotopic composition and elemental content, approximately 5-7 mg of dry leaf sample
340 or 12-15 mg of dry and sieved sediment sample were weighed into tin vials and measured with
341 an Isoprime 100 Isotope Ratio Mass Spectrometer interfaced with a Micro Vario Elemental
342 Analyzer (Elementar Americas, Mt. Laurel, NJ). The sample weights were recorded to the
343 nearest microgram. The majority of the samples were run in duplicate to check for instrument
344 precision. Laboratory standards were periodically used throughout each run (approximately
345 every 24 samples) to identify potential instrument drift and to correct for measurement offset
346 error. Elemental content (%N or %C) was calculated by comparing the peak area of each
347 unknown sample to a standard curve of peak area vs. known %N content. Molar C:N was
348 determined by dividing the measured element content by molar mass.

349 *2.4.3. Below-ground leaf decomposition*

350 In July 2012, locally-sourced recently fallen leaves were collected and processed using
351 the same methods as described for above-ground litter bags. Three leaves were sealed in a 5 cm

352 x 10 cm fiberglass mesh bag with 1 mm^2 pores. For each quadrat, an approximate 20 cm cut was
353 made into the forest floor at three locations. One litter bag was buried within each slice with the
354 long end vertical. The top of the bag was approximately 5 cm from the surface, with leaf
355 material buried between 5-15 cm. At each quadrat, one litter bag was collected at six months,
356 one year, and 22 months after burial. Extraction of the litter bags often involved cutting
357 mangrove root ingrowth; any mangrove roots inside the bag were separated from the
358 decomposed leaf material as carefully as possible. The extracted litter was cleaned and dried as
359 described for the 2013 above-ground litter bags, and initial to final weights were calculated as
360 described above.

361 *2.4.4. Green leaf C:N*

362 Green leaves were directly harvested from mangrove trees in ambient and
363 +moderate N:P quadrats. Three to 5 individual trees were sampled (3 leaves per tree) and kept
364 cool under transport. The leaves were rinsed with distilled water and placed in a drying oven at
365 45°C until a constant weight was reached. The samples were combined per tree and ground
366 using a Wiley-grinding mill with a #20 sieve. Triplicate subsamples were analyzed for C:N ratios
367 using a Flash EA-112 CHNSO analyzer (U.S. EPA Atlantic Coastal Environmental Sciences
368 Division, Narragansett, RI). The trees were averaged within a quadrat to report a molar C:N
369 value.

370 *2.5. Data analyses*

371 All statistical analyses were performed with a mixed effects model (SAS version 9.3,
372 SAS Institute, Cary, North Carolina, USA). The same model was applied for both randomized
373 complete (full sampling) or incomplete (restricted sampling) block designs. Differences in bulk
374 density, percent organic matter and C:N ratios among treatments were evaluated using a two-way

375 analysis of variance (ANOVA) with treatment as a fixed effect and block as a random effect. A
376 3-way ANOVA with treatment as a fixed effect and both time and block as random effects was
377 performed for substrate CO_2 flux values and the percent of remaining mass for above- and
378 below-ground litterbags. The values for decomposition (percent mass remaining) rates and
379 sediment percent organic matter were arcsine transformed to meet normality and homogeneous
380 variance assumptions. Results are reported to a significance level of 0.05. If a significant
381 treatment effect was found, a post-hoc Tukey's HSD pair-wise comparison test for variance was
382 performed with alpha set at 0.05.

383 **3. Results**

384 *3.1 Sediment properties*

385 The mangrove forest floor is heterogeneous, as demonstrated by the range of bulk
386 density, organic matter content, and percent C and N within and among blocks of the mangrove
387 forest (Table 1). Sediment bulk density ($F_{2,4} = 0.25$, $p = 0.79$), organic matter ($F_{2,4} = 0.31$, $p =$
388 0.75) and C:N ($F_{1,2} = 2.49$, $p = 0.26$) did not differ significantly among treatment levels. Total C
389 accounted for 25 to 49% of sediment organic matter content. Across all quadrats, sediment bulk
390 density and OM content were negatively correlated ($F_{1,7} = 11.64$; $p = 0.01$; Figure 2).

391 *3.2 CO_2 flux*

392 CO_2 flux rates from the forest floor in March 2013 (following 72 weeks of nutrient
393 enrichment) ranged from 1.15 to 1.81 μmol CO_2 m^{-2} s^{-1} (or, 3.0 to 4.8 g CO_2 m^{-2} $year^{-1}$) (Figure
394 3). While there was a significant blocking effect, CO_2 flux rates did not differ among treatments
395 ($F_{2,17} = 1.11$, $p = 0.35$). Forest floor CO_2 flux rates after a 30-week recovery period without
396 nutrient enrichment (May, 2014) ranged from 0.58 to 1.55 μmol CO_2 m^{-2} s^{-1} (or, 1.5 to 4.1 g CO_2
397 m^{-2} $year^{-1}$) and were significantly lower than rates measured during the nutrient enrichment

period (March, 2013) ($F_{2,4}$ = 11.96, p = 0.001). There was also no difference among treatments for the follow-up CO_2 measurement.

3.3 Litter decomposition

Incubation time was a significant factor in above-ground leaf litter mass loss ($F_{3,28}$=42.09, p < 0.0001; Figure 4) and litter C:N ($F_{3,28}$ = 42.17; p < 0.0001; Figure 5). Differences among blocks was also significant ($F_{2,28}$ = 4.37, p = 0.02). In the first month of incubation, 0 to 30% of above-ground leaf litter mass was lost under ambient and +high N:P nutrient treatments, and 15 to 40% of litter mass was lost under +moderate N:P nutrient treatment. The percent of above-ground litter mass remaining ranged from 36 to 56% by 90 days. Nutrient treatments had no significant effect on litter decomposition values ($F_{2,28}$=0.66, p = 0.52) nor corresponding time to 50% mass decay ($F_{2,4}$=0.69; p = 0.55). The calculated time for above-ground litter decay ranged from 61 to 110 days, with an average of 79 days (Table 2). The average decay constant (k) was 0.009 d^{-1} (range 0.006 to 0.011 d^{-1}).

More than 80% of initial leaf material had decomposed within the first six months of below-ground incubation (Figure 6). There was little decomposition of the remaining buried leaf material between six months and one year. At the final below-ground litter bag collection (22 months after burial), the remaining leaf material had disintegrated into small pieces (< 0.25 cm^2) and was completely integrated with the fine root matrix. Thus we determined all leaf material to be completely decomposed after 22 months of shallow burial for all quadrats. There was no significant effect of nutrient treatment on below-ground decomposition ($F_{2,12}$ = 0.66, p = 0.52); however there was a significant block effect ($F_{2,12}$=4.37, p = 0.02).

3.4 Green leaf C:N

420 Molar C:N content of green leaves collected at the end of the two-year experiment was
421 lower under +moderate N:P fertilization compared with ambient treatment ($F_{1,2}$ = 114.9, p =
422 0.009; figure 7). There was a significant different among the blocks ($F_{2,2}$ = 185.54; p = 0.005).

423 **4. Discussion**

424 The purpose of this experiment was to determine if organic C mineralization rates
425 associated with a mangrove forest floor, and as described through substrate CO_2 flux and litter
426 decomposition assessment, would be modified under anthropogenic nutrient enrichment. Within
427 a single mangrove stand there was considerable variation in terms of edaphic structure (sediment
428 bulk density, OM content) and environmental conditions (tidal inundation duration and
429 frequency) along the coastal fringe that resulted in a significant difference in response variables
430 among blocks (see supplemental information). Yet considering these differences of the
431 experimental blocks, treatments, and time periods, rates of substrate CO_2 flux and above-ground
432 OM decomposition were remarkably consistent.

433 An additional goal of this study was to conduct an intermittent pulse application of
434 aqueous fertilizer over a two-year period and compare the results of this method with past
435 fertilization studies. Much of the global knowledge of the role of nutrients for mangrove C
436 dynamics are based on studies employing buried slow-release fertilizer, often in stressed
437 environments due to nutrient limitation and/or salinity stress (Feller et al. 1999, 2007, Lovelock
438 et al. 2015, Keuskamp et al. 2015). This study attempts to compare the results of those works
439 with similar measurements from a relatively non-stressed fringe mangrove undergoing nutrient
440 addition that mimics enriched sheet flow or tidal inundation. While care was taken to minimize
441 loss of the added fertilizer, some loss was inevitable given nutrient exchange under diel tidal
442 cycles. Most of the recovered labeled fertilizer (at least 16.6 to 23.4% at 10 months following

443 ^{15}N tracer addition) was found in fine mangrove roots, indicating that the below-ground roots
444 "outcompeted" surface- and below-ground microbes for the added N. Nutrient uptake by fine
445 roots is a typical response to low nutrient availability (Castañeda-Moya et al. 2011, Cormier et
446 al. 2015); however McKee et al. (2007) measured greater rates fine root production in fringe
447 mangrove sites fertilized with P but not N. This study did not examine root production rates, but
448 based on dominate ^{15}N uptake within fine roots, further study of the effects of N fertilization on
449 root production at this site would be warranted.

450 Given the results from the ^{15}N tracer data, an appropriate concern is whether the benthic
451 microbial heterotrophs encountered the additional N given tidal inundation at the fringe site.
452 Work by Corredor and Morrell (1994) and Whigham et al. (2009) show immediate enhanced
453 denitrification rates in mangrove sediments in response to nitrate enrichment. Corredor and
454 Morrell (1994) measured denitrification rates in a mangrove forest along a nutrient gradient
455 extending from a sewage treatment plant. Sediment denitrification rates decreased at greater
456 distance from the sewage source, although potential denitrification rates (with available
457 substrate) were maintained. Whigham et al. (2009) found higher rates of denitrification for
458 several days after monthly doses of aqueous nitrate in a Florida stunted *Avicennia germinans*
459 forest. Based on the rapid positive response by the denitrifier community under both scenarios,
460 it may be possible the added nutrients were effectively removed, thereby preventing a long-term
461 pool of available N.

462 We were limited in our ability to conduct a complete sample analysis for all quadrats. A
463 more comprehensive understanding of the drivers of C cycling under the aqueous enrichment
464 scenario requires enhanced component sampling and modeling of the added nutrients to
465 determine how much aqueous fertilizer was retained.

466 *4.1 Substrate CO_2 flux*

467 *In situ* measurements of CO_2 flux from the forest floor encompass microbial activity,
468 benthic algal metabolism, tree root respiration, and possibly other burrowing animal respiration.
469 For the purpose of this study we must assume all factors besides microbial activity remain
470 consistent between time periods and within blocks. Average CO_2 flux from the mangrove forest
471 floor in this study (1.24 µmol CO_2 m^{-2} s^{-1}) was within the range of a global survey of dwarf and
472 fringe mangroves (0.25 to 2.97 µmol CO2 m^{-2} s^{-1}; Lovelock 2008) .

473 Minimal microbial response to nutrient enrichment is confirmed by previous work in
474 mangrove systems. Lovelock (2008) notes that anthropogenic land use was not the primary
475 driver of sediment respiration rates in a global survey of dwarf and fringe mangrove sites.
476 Kristensen et al. (2011) likewise found no effect of nutrient enrichment on OM mineralization
477 (except see below). One possible explanation of a lack of response to nutrient enrichment is the
478 "nitrogen mining theory" (Craine et al., 2007), which postulates that N-limited microbial
479 communities break down refractory OM to acquire nitrogenous compounds; when the N
480 limitation is lifted, the microbes have less energetic incentive to continue the decomposition
481 process. Thus N fertilization may actually inhibit microbial decay of highly refractory organic
482 substrate, which constitutes the bulk of mangrove peat (Middleton and McKee, 2001). In
483 support of this concept for mangrove systems, Keuskamp et al. (2012) found that N enrichment
484 alone had no effect on the rate of sediment OM mineralization in mangrove sites, but microbial
485 activity spiked upon the co-introduction of labile C (glucose) and N enrichment. They
486 concluded that substrate OM mineralization was energy limited due to the refractory OM matrix,
487 which could be further inhibited by high tannin content (Robertson, 1988). Kristensen et al.
488 (2011) found no effect of nutrient enrichment from a sewage site on mangrove microbial

processes, but a greater input of labile organic C from an algae bloom "of anthropogenic origin" enhanced CO_2 flux in the intertidal zone. Thus, nutrient enrichment may affect sediment mineralization rates via a cascade effect, where nutrient enrichment enhances algae production, which delivers labile organic C to the mangrove sediments, which stimulates heterotrophic decomposition.

Based on these works, a broader examination of the nutrient mining theory for benthic mangrove response to nutrient enrichment is needed, especially with the consideration that labile organic substrate may be linked with nutrient enrichment via primary-treatment sewage and septic systems, agriculture practices that utilize urea or animal wastes, or enhanced organic material production in eutrophic coastal waters.

4.2 Leaf Litter Decomposition

The rate of litter decomposition depends on the quality and availability of the substrate as well as several other factors including macroinvertebrate (shredder) activity, physical shearing forces (e.g., tidal submersion), temperature, oxidation state of the local environment, and resource limitations of the decomposer community such as nutrient availability (Benner and Hodson, 1985; Mackey and Smail, 1996; Twilley et al., 1997). Li et al. (2018) demonstrated that mangrove production and remineralization rates of organic material depended on site location. Thus a comparison of litter decomposition rates among systems is somewhat difficult without all biological and environmental factors taken into consideration. Above-ground litter decomposition rates from this study fit within previously reported above-ground litter decomposition values from other fringe mangrove systems, whether natural (Twilley et al., 1986; 1997; Aké-Castillo et al., 2006) or provided with additional nutrients (Tam et al. 1998). These studies suggest that nutrient enrichment does not necessarily play a dominant role in mangrove

512 above-ground litter decomposition, and the factors described above are important covariates to
513 be considered for an accurate prediction of decomposition rates. For example, Keuskamp et al.
514 (2015) reported that physical setting (Florida vs. Belize) was a better predictor of litter
515 decomposition rates than nutritional status, presumably due to other environmental factors.

516 The process of OM decomposition can be described in two phases: (1) an initial leaching
517 stage, where soluble compounds and labile material are easily dissolved within the aqueous
518 medium; (2) a microbial colonization and slow decomposition stage, where refractory
519 compounds such as lignins are chemically broken down by the microbial community (Chale,
520 1993; Davis et al., 2003). The curves of above-ground litter mass loss were generally linear
521 throughout this study, suggesting that the decomposition stage had not fully matured to the slow
522 process of refractory material decomposition. The time to reach 50% decay ranged from 61 to
523 110 days, which is comparable to litter decomposition in a similar fringe mangrove studied by
524 Aké-Castillo et al. (2006; Table 3). This t_{50} range was faster than that reported by Twilley et al.
525 (1986) in basin mangroves, which are less regularly flushed than fringe mangroves (Lugo and
526 Snedaker 1974). The range of t_{50} was much higher in Tam et al. (1998) compared with this
527 study, but those decomposition rates under wastewater application were not significantly
528 different than control sites (Tam et al. 1998). Tam et al. (1998) report rainfall levels an order of
529 magnitude greater than received at Jobos Bay, which suggests that the freshwater input has a
530 positive influence on above-ground litter decomposition.

531 Below-ground leaf litter became fully decomposed and integrated within the mangrove
532 root complex within two years of burial. Middleton and McKee (2001) reported 27 $\pm$ 5% mass
533 remaining of buried leaves after 230 days of incubation, which was similar to the values at one
534 year of incubation in this study. As we used fiberglass mesh litterbags, this rate precluded the

effect of macroinvertebrate shredding, which would have enhanced the decomposition rate. The relatively fast break-down of buried mangrove leaves is an indication that mangrove leaf litter is not a primary component of mangrove peat material; rather roots contribute the majority of the structural mangrove peat (Middleton and McKee, 2001). Castañeda-Moya et al. (2011) described a relationship between fine root biomass and turnover rates in relation to soil nutrients, but hydroperiod was also a main co-factor. Therefore more examination of root turnover under nutrient loading scenarios that control for other environmental variables would be a valuable contribution to the body of work on mangrove carbon dynamics.

Under both ambient and fertilized treatments, mangrove litter C:N decreased as above-ground decomposition progressed. Additionally, C:N was significantly lower for fertilized litter bags, which suggests that while the amount of ^{15}N labeled fertilizer retention was not very high, there may have still been a functional effect. The main driver of this nutritional change is N immobilization by microbial colonization of the OM substrate. Microbial colonization and break-down of organic substrate is linked to exo-enzyme secretion (Keuskamp et al., 2015b) and nitrogen fixation (Holguin et al., 2001), which enrich the OM-microbial complex with nitrogenous compounds. Plant tannins may bind available N (Kraus et al., 2003), further immobilizing the N in the OM matrix.

5. Conclusions

The relationship between production and loss of organic material is a significant factor in maintaining the mangrove substrate (Bouillon et al. 2003, McKee et al. 2007, Bouillon et al. 2008, Kristensen et al. 2008) and may be altered by nutrient enrichment (Feller et al. 1999). Under a two-year delivery of N and P to a fringe mangrove floor in aqueous pulses we found no response by the benthic decomposer activity as measured by CO_2 flux and above- and below-

ground decomposition, suggesting that the microbial community is not strongly limited by available N and/or P at this coastal fringe site. Below-ground roots may be holding the majority of retained fertilizer and "out-competing" the microbial decomposer community, which may enhance production over decomposition rates at this site. A longer-term study is needed to compare experimental aqueous nutrient delivery with the foundational knowledge for mangrove carbon dynamics based on buried fertilizer studies. While it appears that water-based nutrient pollution does not affect organic C decomposition at this fringe mangrove site in the short-term, more work is needed to understand the role of labile organic C in conjunction with nutrient enrichment for benthic respiration activity in a variety of mangrove environmental settings.

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769 S. O., 2008. Characterizing Jobos Bay, Puerto Rico: A watershed modeling analysis and
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774 **Fig. 1.** Satellite image of Jobos Bay, located in southeastern Puerto Rico. The
775 boundaries of the Jobos Bay National Estuarine Research Reserve are outlined in red. A
776 white arrow indicates the location of the nutrient enrichment experiment at offshore keys,
777 approximately 3 km from the mainland (image credits: NOAA-NERR 2015, Zitello et al.
778 2008).
779

780
781 **Table 1**. Edaphic characterization of experimental quadrats grouped within three blocks of a fringe mangrove forest.

	Block 1			Block 2			Block 3		
	Control	+Moderate N:P	+High N:P	Control	+Moderate N:P	+High N:P	Control	+Moderate N:P	+High N:P
Bulk density (g/cm^3) (n = 2)	0.53 (0.05)	0.48 (0.07)	0.38 (0.03)	0.17 (0.03)	0.18 (0.02)	0.23 (0.02)	0.23 (0.02)	0.35 (0.05)	0.28 (0.03)
Organic matter (%) (n = 2)	12.9 (1.4)	15.4 (2.5)	26.8 (14.4)	47.6 (14.9)	45.4 (5.1)	36.3 (0.6)	18.2 (4.7)	22.4 (7.2)	24.0 (2.1)
Total C (% by weight) (n = 3)	3.34 (0.60)	5.46 (0.79)	ND	19.46 (0.88)	20.66 (1.85)	ND	8.97 (1.58)	10.90 (0.42)	ND
Total N (% by weight) (n = 3)	0.13 (0.03)	0.19 (0.03)	ND	0.74 (0.03)	0.77 (0.08)	ND	0.34 (0.05)	0.34 (0.02)	ND
Molar C:N (n = 3)	32.0 (1.8)	33.4 (1.0)	ND	30.6 (0.4)	31.4 (1.2)	ND	30.9 (1.7)	37.6 (3.5)	ND

782
783 Values are mean $\pm$ 1 S.E. "ND" indicates that the data are not available. There was no effect of nutrient treatment on sediment bulk
784 density ($F_{2,4} = 0.25$; $p = 0.79$) or percent organic matter ($F_{2,12} = 1.07$; $p = 0.38$); however there was a block effect for both variables
785 (see supplemental).
786

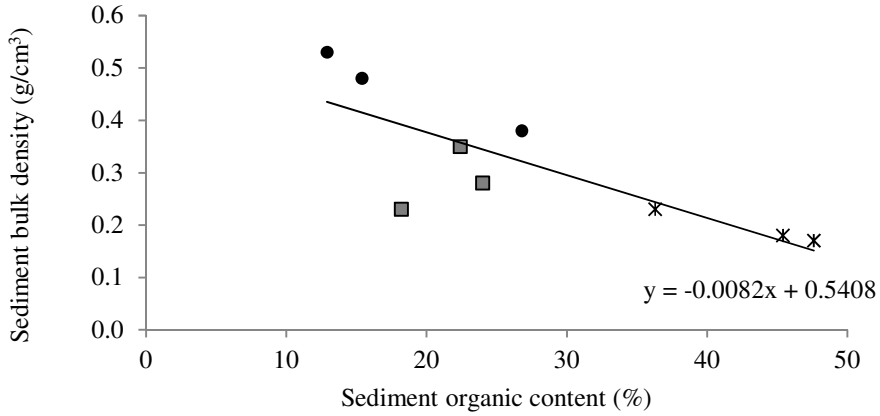

Fig. 2. Sediment bulk density (dry mass by volume) and percent organic content (ash-free dry weight content) of the surface organic layer of the mangrove forest sediment (0 to 5 cm) Data were averaged from collections in July 2011 and October 2013. Circles represent block 1, crossed lines represent block 2, squares represent block 3. A linear regression was applied for all nine quadrats ($F_{1,7} = 11.64$; $p = 0.01$)

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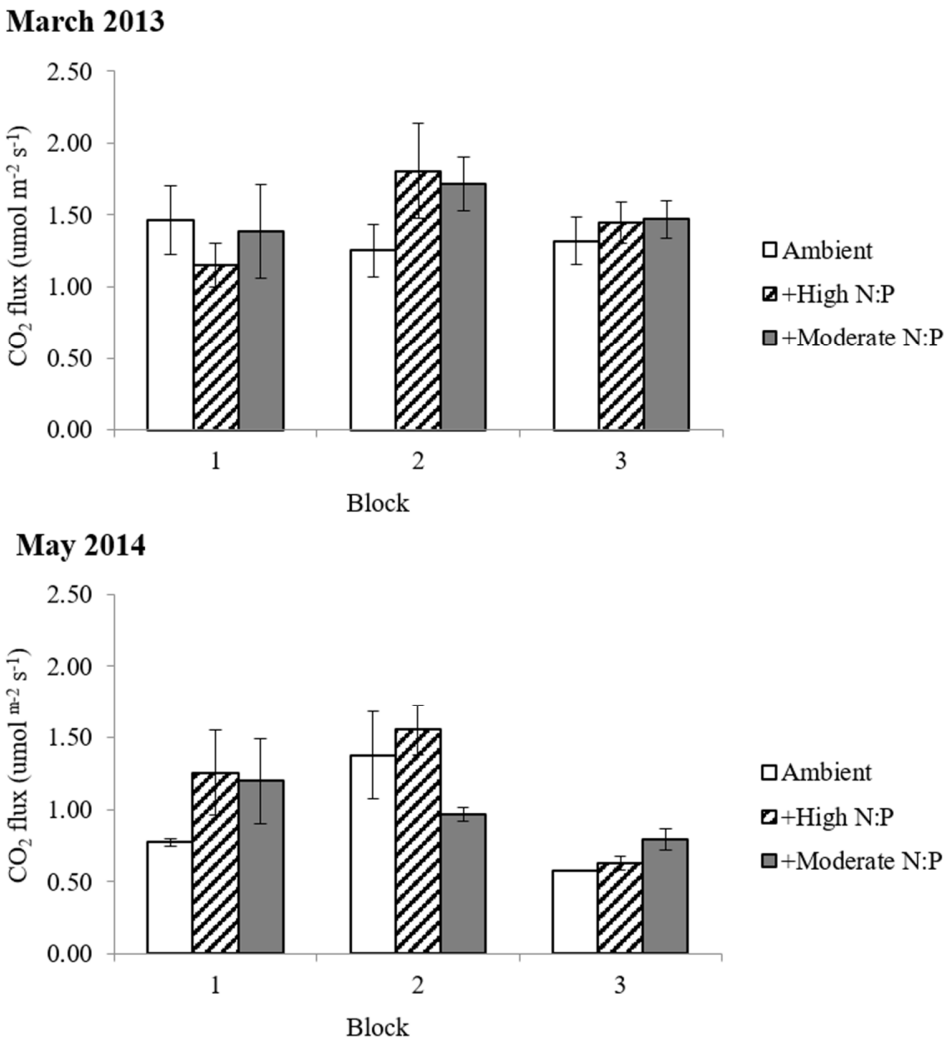

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799 **Fig. 3.** Substrate CO_2 flux from the unflooded forest floor at two time periods: (A)
800 March 2013, 72 weeks of nutrient enrichment; (B) May 2014, 30 weeks after nutrient
801 enrichment ended. There was a significant difference between the sampling dates ($F_{2,17}$ =
802 11.96, p = 0.0006). Values are means $\pm$ 1 S.E.

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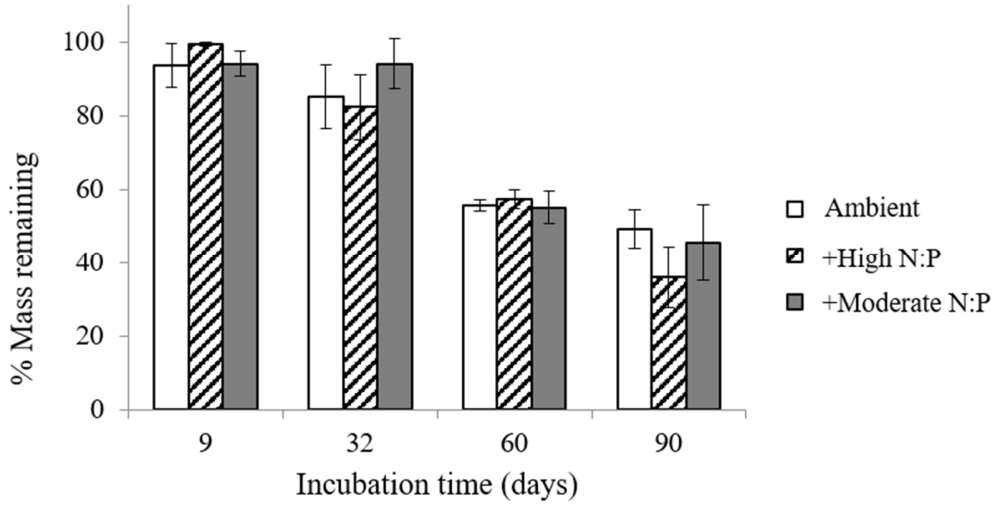

804
805 **Fig. 4.** Percent mass remaining for above-ground mangrove leaf litter decomposition.
806 Above-ground leaf litter decomposition under three nutrient conditions (described in
807 Methods) during July – October 2013. Leaf litter was incubated in mesh bags on the
808 forest floor and subjected to tidal flow. Linear regressions were used to determine the
809 number of days for 50% decay (t_{50}; see supplemental information). Error bars indicate
810 $\pm$ 1 S.E.
811

812

813 **Table 2.** 50% decay rate (t_{50}) as determined from a linear regression of litter decay (see
814 supplemental) and decay constant (k) calculated from the exponential decay curve $X = X_o e^{-kt}$
815 with time (t) set at the day that litter decay reached 50% of initial weight during the incubation
816 period. There was no effect of nutrient treatment on t_{50} values ($F_{2,4}$=0.69, p = 0.553).

Treatment	Block	t_{50}	k
Control	1	92	0.0075
	2	88	0.0079
	3	71	0.0098
Agriculture	1	82	0.0084
	2	76	0.0091
	3	61	0.0114
Urban	1	110	0.0063
	2	66	0.0105
	3	65	0.0107

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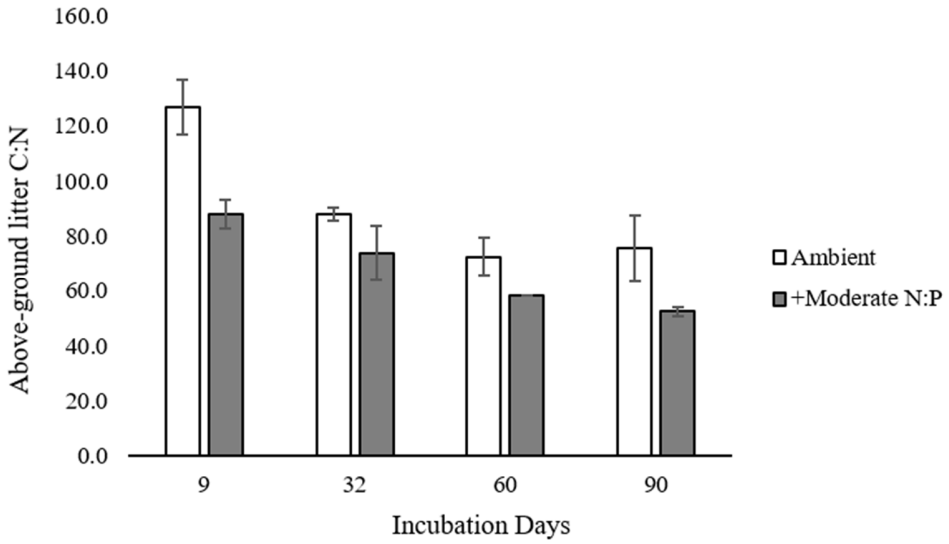
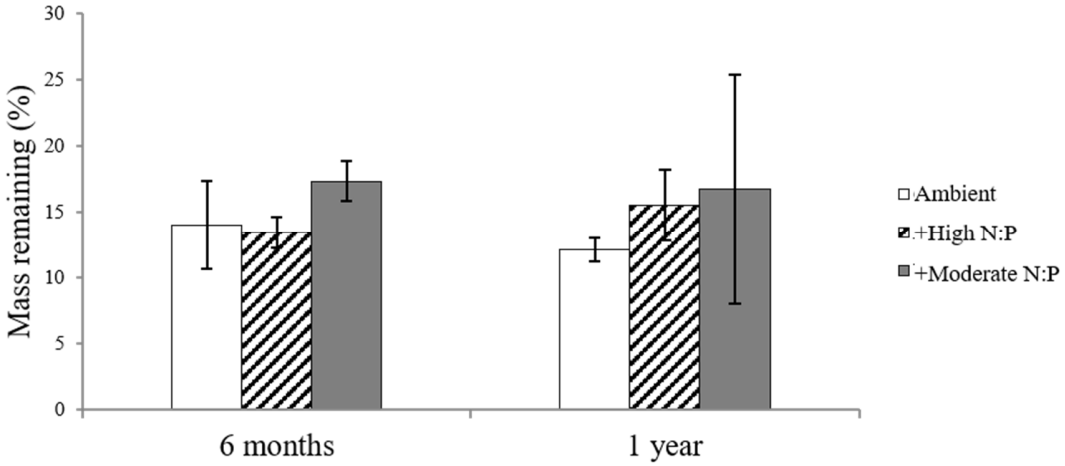


Fig. 5. Molar C:N ratio of above-ground decaying leaf litter under the +Moderate N:P fertilization treatment (grey bars) and ambient conditions (white bars). Samples were collected from litter bags that had incubated on the forest floor from July to October 2013. Incubation time had a significant effect ($F_{3,16} = 18.82$, $p < .0001$) on litter C:N under both nutrient regimes. Litter C:N values were significantly different between ambient and enriched plots ($F_{1,16} = 5.69$, $p = 0.03$).



841

842 **Fig. 6.** Percent mass remaining for below-ground mangrove leaf litter decomposition. The litter
843 was buried at 5 to 15 cm depth in July 2012. Values are means across blocks (n = 3). Error bars
844 indicate +/- 1 S.E. Litter material recovered at 22 months past the burial date was integrated
845 with fine root material and was considered fully degraded.
846

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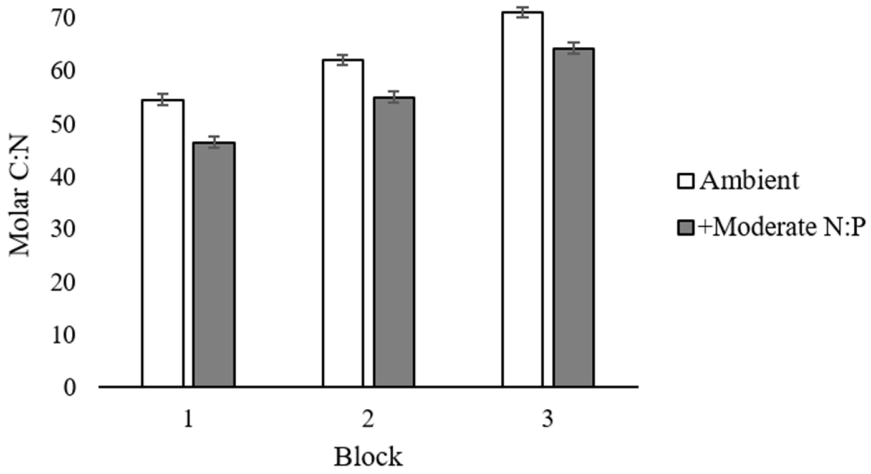

848

849 **Fig 7.** Molar C:N values in green leaves collected directly from trees located in ambient (white
850 bars) or +moderate N:P enriched (grey bars) at the end of the two-year experiment (October,
851 2013). Leaves collected from 3-5 trees per quadrat were pooled. Error bars indicate $\pm$ 1 S.E.
852 Leaf C:N was significantly different across treatments ($F_{1,2}$ = 114.9, p = 0.009) and blocks ($F_{2,2}$ =
853 185.54; p = 0.005)

Table 3. Reported values of above-ground litter decomposition studies (number of days to 50% decay rate (t_{50}) and decay constant (k)) from mangrove systems across the world with classification (Lugo and Snedaker 1974). All studies used the litter bag method to measure decomposition. Decay constants (k) that were not reported in the study were calculated using the exponential decay curve $X = X_o e^{-kt}$ with time (t) set at the day that litter decay reached 50% of initial weight during the incubation period.

Study location	Mangrove type/species	Nutrients added	t_{50}	k (day $^{-1}$)	Reference
Shenzhen, China	*Fringe: K. candel*	Wastewater	13	0.052	Tam et al. (1998)
	Fringe: A. corniculatum	Wastewater	48	0.015	
Mexico	*Fringe: R. mangle*	None	70 to 144	0.0048 to 0.0084	Aké-Castillo et al. (2006)
Ecuador	*Riverine: Rhizophora spp.*	None	43 to 231	0.003 to 0.016	Twilley et al. (1997)
Florida	*Basin: R. mangle*	None	98 to 165	0.0042 to 0.0071	Twilley et al. (1986)
Florida	*Scrub: A. germinans*	Buried fertilizer	52 to 58	0.009	Simpson et al. (2020)
Puerto Rico	*Fringe: R. mangle*	Spray fertilizer	61 to 110	0.006 to 0.011	This study