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Short- and long-term effects of nutrient enrichment on microbial exoenzyme activity in mangrove peat

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ABSTRACT

Mangroves receive increasing quantities of nutrients as a result of coastal development, which could lead to significant changes in carbon sequestration and soil subsidence. We hypothesised that mangrove-produced tannins induce a nitrogen (N) limitation on microbial decomposition even when plant growth is limited by phosphorus (P). As a result, increased N influx would lead to a net loss of sequestered carbon negating the ability to compensate for sea level rise in P-limited mangroves. To examine this, we quantified the short- and long-term effects of N and P enrichment on microbial biomass and decomposition-related enzyme activities in a *Rhizophora mangle*-dominated mangrove, which had been subjected to fertilisation treatments for a period of fifteen years. We compared microbial biomass, elemental stoichiometry and potential enzyme activity in dwarf and fringe-type *R. mangle*-dominated sites, where primary production is limited by P or N depending on the proximity to open water. Even in P-limited mangroves, microbial activity was N-limited as indicated by stoichiometry and an increase in enzymic activity upon N amendment. Nevertheless, microbial biomass increased upon field additions of P, indicating that the carbon supply played even a larger role. Furthermore, we found that P amendment suppressed phenol oxidase activity, while N amendment did not. The possible differential nutrient limitations of microbial decomposers versus primary producers implies that the direction of the effect of eutrophication on carbon sequestration is nutrient-specific. In addition, this study shows that phenol oxidase activities in this system decrease through P, possibly strengthening the enzymic latch effect of mangrove tannins. Furthermore, it is argued that the often used division between N-harvesting, P-harvesting, and carbon-harvesting exoenzymes needs to be reconsidered.

1. Introduction

Mangrove ecosystems are commonly found in tropical and subtropical coastal zones, where they are of major importance to local nutrient- and carbon-cycling (Alongi, 1996). In the absence of soil elevation, rising sea levels lead to dieback of mangroves resulting from increased inundation times. In systems where sediment input is not substantial, survival of mangroves depends mostly on the build-up of peat (McKee, 2011), resulting from the imbalance between primary production and decomposition. Nutrient enrichment in these systems can influence both of these processes. Eutrophication, therefore, may have unforeseen effects on mangrove stability (McKee et al., 2007), leading to habitat loss if soil accumulation is negatively affected.

Decomposition is catalysed by several key exoenzymes that allow for extracellular conversion of complex organic matter into simpler products, such as glucose, amino acids, and phosphate. Mangrove litter, especially from *Rhizophora* spp., contains large amounts of dissolvable and non-dissolvable tannins (Alongi, 1987; Maie et al., 2006; Zhang et al., 2010). As protein-binding phenolic compounds, tannins can inhibit microbial activity and lower nutrient mobilisation via substrate deprivation and enzyme inhibition (Schimel et al., 1996; Kraus et al., 2003; Joanisse et al., 2007). The immobilisation of exoenzymes in tannin-rich soils decreases

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their activity (Ximenes et al., 2011), resulting in reduced return of degradation products per unit of investment of nitrogen (N), carbon (C), and energy. This induces energy and/or N limitation in microorganisms producing these enzymes, thereby suppressing microbial decomposing activity.

We hypothesised that decomposer microorganisms in mangroves are ultimately N-limited, due to tannin-protein complexation. If primary production is phosphorus (P)-limited, as is common for mangroves in carbonate-rich environments, tannin-induced N limitation for microorganisms will lead to a differential nutrient limitation (DNL) for plants versus microbial decomposers. DNL therefore results in nutrient-specific biogenic controls of soil level in mangrove ecosystems. In such systems, enrichment in the plant-limiting nutrient (P) would result in net peat accumulation, whereas enrichment in the microbe-limiting nutrient (N) would result in net peat loss. The latter poses a large threat to mangrove systems on a global scale, because many coastal systems are experiencing increasing anthropogenic N inputs (Howarth and Marino, 2006).

Exoenzymes produced by microorganisms catalyse the rate-limiting step in nutrient-cycling and decomposition of the organic matter contained in peat (Sinsabaugh, 1994; Freeman et al., 2004), and their activities correlate with the rate of soil organic C decay and microbial nutrient demand (Sinsabaugh et al., 2009). Hydrolytic enzymes are specific in the reactions they catalyse and are frequently used to determine whether decomposition is limited by N or P (Sinsabaugh and Moorhead, 1994). These enzymes are not capable of breaking down tannins. The much less specific oxidative enzymes are capable of breaking down tannins and other polyphenolic compounds (Freeman et al., 2004; Limpens et al., 2008). The relative activities of hydrolytic and oxidative enzymes are a major determinant of peat formation (Freeman et al., 2001). A low relative activity of oxidative enzymes leads to enrichment with polyphenolic compounds which strongly promotes peat formation while a high relative oxidative enzyme activity depletes peat in polyphenolic compounds which can lead to rapid disappearance of historically formed peat (Freeman et al., 2001).

To gain insight into the effects of nutrient enrichment on enzyme-mediated decomposition processes in mangrove systems, we used two complementary approaches: i) tracking the short-term impacts of nutrient enrichments in laboratory incubations to assess the response to nutrient and C-enrichment without the confounding effects of plant–soil interactions, and ii) measuring the effects of long-term nutrient fertilisation in field experiments to examine the long-term, *in situ* consequences of nutrient enrichment on oxidative and hydrolytic enzyme activities in the context of nutrient competition between microorganisms and plants. In addition to quantifying potential enzyme activities, we compared microbial biomass, elemental stoichiometry, and metabolic activity between the different fertilisation treatments to test the hypothesis that tannin production creates an N limitation for microbial decomposers regardless of the limitations on primary production.

The present study was conducted at Twin Cays, a mangrove-dominated oceanic island group in the Caribbean Sea, 16 km off the coast of Dangriga, Belize. The islands consist of mangrove-derived peat that is up to 10 m thick (McKee et al., 2007). The dominant mangrove species on the island, *Rhizophora mangle*, shows a clear zonation with respect to growth form and nutrient limitation: Trees on the inland parts of the island show stunted growth and are strongly P-limited (dwarf zone), while trees near the fringes of the island are much taller, and are generally N-limited, depending on their proximity to open water (fringe zone) (Feller et al., 2002).

Earlier studies have attempted to quantify the effects of nutrient enrichment on peat decomposition in mangrove systems, using decomposition rates of roots (McKee et al., 2007) or tensile strength loss of cotton-strips (Feller et al., 2002) as indicators. These studies suggested that decomposition was either P-limited (Feller et al., 2002) or not sensitive to nutrient enrichment (McKee et al., 2007). However, McKee et al. (2007) observe peat decline upon N fertilisation, possibly due to increased decomposition rates (Lovelock et al., 2011). A mechanistic approach that employs the measurement of both hydrolytic and oxidative exoenzymes involved in the breakdown of organic matter provides a means to reconcile these seemingly contradictory results.

Following from our hypothesis, we expected microbial decomposition to be ultimately N-limited due to the effects of mangrove tannins. This would mean that the dwarf zone, where primary production is P-limited, has a DNL, while the fringe zone, where primary production is N-limited, does not. When microbial decomposers and primary producers are both limited by the same nutrient, the decomposition response comprises of direct effects and plant-mediated effects (i.e. through changes in tannin production or litter quality). The combined study of short- and long-term effects of nutrient amendments on enzyme production allows for differentiation between these direct and plant-mediated responses, indicating their relative importance. The results from this study can be used to evaluate the use of enzyme activities to assess microbial nutrient limitations (Sinsabaugh et al., 2008), and to qualify the potential consequences of eutrophication with respect to peat decomposition in mangroves.

2. Materials and methods

2.1. Study site and field experiment

Soil and water samples were collected at Twin Cays, Belize (16°49′N, 88°06′W). An extensive description of the hydrology, climate, primary production, and soil properties can be found in Feller (1995); Feller et al. (1999, 2002); McKee et al. (2007); Lee et al. (2008); Feller et al. (2009).

At the date of sampling, the *R. mangle*-dominated sites had been fertilised with either N or P for over 15 years using the method described in Feller (1995). In short, the fertiliser application consisted of the semiannual burial of two pieces of dialysis tubing filled with 150 m of either urea for N fertilisation or triple superphosphate for P fertilisation at opposing sides close to the base of each fertilised tree. This procedure led to a total annual amendment of 335 m NH_3 in the N treatment and 452 m PO_4 in the P treatment.

A detailed explanation of the lay-out of the long-term fertilisation experiment is described by Feller et al. (2002). Transects with a maximum length of 50 m perpendicular to the coastline were established at three randomly chosen positions (Feller, 1995). At each position, three parallel transects with a lateral distance of about 10 m were established and a fertilisation treatment (control, P fertilisation, and N fertilisation) was randomly assigned to each of these transects. Each transect consisted of a fringe zone where *R.*

Table 1
General properties as measured at *Rhizophora mangle*-dominated stands on a mangrove-covered island in the Caribbean sea near Belize ($N = 9$). Numbers following ± represent standard errors.

Property	Dwarf	Fringe	Unit
Tree height	0.99 ± 0.16	4.72 ± 0.36	m
Tree density	1.3 ± 0.1	1.0 ± 0.3	individuals m^{-2}
pH	6.7 ± 0.1	6.5 ± 0.1	–
Temperature	19.7 ± 0.6	16.4 ± 0.2	°C
Bulk density	0.92 ± 0.1	0.92 ± 0.1	g soil DW cm^{-3}

mangle trees showed tall (5–6 m) growth, a narrow transition zone with intermediate tree heights, and a dwarf zone, where *R. mangle* trees showed stunted growth with distinctively lower tree height (<1.5 m) and density.

Some general soil properties of the dwarf and fringe zones are given in Table 1. The two zones did not differ significantly with respect to pH and soil bulk density, but salinity and temperature were lower in the fringe sites.

2.2. Soil level calculations

At each site, the level of inundation at a specific moment was measured using a ruler. Soil elevation relative to Mean Lower Low Water (MLLW) was calculated using the TideCal 10 (Kaleberg Symbionts, 2010) OS X port of Xtide v 2.10 (Flater, 2008) with the tide_db v1.07 harmonics file (Depner, 2003) using the Belize City measurement station as a reference.

2.3. Sample collection and processing

In spring 2012, average tree height and density were measured in a 2 × 2 m quadrant with the fertilised tree as the centre. Soil and porewater samples were taken approximately 10 cm from the base of a tree. Porewater was collected using 10 cm long rhizons (Eijkelkamp BV, Giesbeek, The Netherlands). Soil samples were taken by extracting cores with a depth of 10 cm using a stainless steel corer with a diameter of 9.6 cm. All samples taken in the field were transported to the lab within 12 h and stored at 3 °C until further processing. After determination of soil bulk density, roots larger than 2 mm were removed, and all samples were manually homogenised before further processing. Gravimetric soil moisture was determined based on moisture loss upon 48 h of oven drying at 70 °C. Soil was freeze-dried and ground at 20 RPM using an MM200 mixer mill (Retsch GmbH, Haan, Germany). Organic C and total N content were determined using an EA/110 elemental CN analyser (InterScience BV, Breda, The Netherlands) after washing with a 32% HCl solution to remove calcium carbonates.

Phenolic compound (i.e. tannin) content was determined using the Folin–Ciocalteu reagens for phenolic compounds following the recommendations of Cicco and Lattanzio (2011) to avoid formation of precipitations. Phenolic compounds were extracted by shaking 15 mg freeze-dried soil with 2 ml extraction fluid at 40 °C for 1 h at 20 rpm, using deionised water as a solvent for soluble phenolic compounds and a 50%–50% methanol–water mixture as a solvent for total phenolic compound extraction. After determination of concentrations of total and soluble phenolic compounds, the insoluble tannin concentration was calculated as the total minus the soluble phenolic compounds. PO_4^{3-}, NH_4^+, NO_3^-, dissolved organic N, and dissolved organic C (DOC) measurements of porewater were conducted using a continuous flow auto analyser (Skalar SA-40, Breda, The Netherlands).

2.4. Microbial biomass and stoichiometry

Microbial biomass and stoichiometry were determined using a fumigation/extraction procedure as described in Keuskamp et al. (2012). After extraction with a 0.5 M K_2SO_4 solution, we measured the amount of extracted N, P and C compounds that were liberated by a 24 h fumigation with ethanol-free chloroform. To estimate microbial C, N, and P content, we divided the liberated amounts of these substances by their respective extractable fractions: 0.38 for C (Vance et al., 1987), 0.54 for N (Brookes et al., 1985) and 0.40 l for P (Brookes et al., 1982).

2.5. Short-term incubation experiment

As described in Keuskamp et al. (2012), soil cores from non-fertilised dwarf sites were incubated for 7 days at a controlled room temperature of 20 °C. Prior to incubation, soils were amended with all possible combinations of labile organic C (as glucose), N (as NH_4Cl), and P (as Na_3HPO_4) yielding a total of eight different treatments. The amount of C added was 5 nmol g^{-1} of fresh soil with molar C:N:P ratio of 50:10:1 to mimic microbial cellular stoichiometry (Fagerbakke et al., 1996).

2.6. Potential enzyme activities

Potential enzyme activity assays of six hydrolytic and two oxidative enzymes were conducted on fresh soil from the various treatments in the dwarf and fringe zones. In addition, potential activities of hydrolytic enzymes were measured in soil from the non-fertilised control treatment in the dwarf zone after 96 h of laboratory incubation with the various nutrient amendments. Following Sinsabaugh et al. (2008, 2009), the measured hydrolytic enzymes can be grouped by their function relative to acquisition of P, N, or C and energy. Alkaline (acid) phosphatase (AP) liberates phosphate through the breakdown of organic phosphate compounds. Leucine aminopeptidase (LAP) and glycine aminopeptidase (GAP) are involved in the acquisition of N-containing compounds. LAP catalyses the hydrolysis of leucine residues from peptides and proteins, and GAP preferentially hydrolyses the terminal N-bond of glycine and alanine. Cellobiohydrolase (CBH) and β-1,4-glucosidase (BG) are involved in the depolymerisation of cellulose, resulting in free glucose for use as a C and energy source. β-N-acetylglucosaminidase (NAG) cleaves N-acetylglucosamine from the fungal and bacterial cell wall components chitin and murein, liberating both C and N. The oxidative enzymes examined, phenol oxidase (POX) and peroxidase (POD), are non-specific and attack C bonds in complex structures such as tannin and lignin.

Potential enzyme activities were measured based on absorbance measurements in 96-well microplates, following a protocol modified from Allison and Vitousek (2004). An enzyme-specific substrate was added to each soil extract to create substrate-saturating conditions. In a given enzymic reaction pNP (in case of AP, CBH, BG and NAG) or pNA (in case of GAP and LAP) is formed, or L-Dopa (in case of POX and POD) is broken down. Soil extracts were prepared by suspending 2 g of soil in 20 ml of TRIS buffered MBL artificial seawater (Cavanaugh, 1956). To measure enzyme activity, eight wells were filled with 150 μl of the suspension and 50 μl of TRIS buffered MBL with or without substrate to serve as reaction and reference wells, respectively. The plates were incubated in the dark at 20 °C for 30–180 min, depending on the enzyme being assayed. During the incubation, the plates were shaken at 600 RPM to keep soil particles in suspension. After incubation, soils were allowed to settle and 100 μl of particle-free solution was transferred to a new plate for measurement of the absorption at 405 nm for pNP- and pNA-containing substrates and at 450 nm for L-Dopa. Absorbance was measured using a SPECTROstar nano photospectrometer (BMG LABTECH, Offenburg, Germany). Product formation was calculated using the difference in absorbance between reaction and reference wells, corrected for change of absorbance through substrate consumption:

$$\Delta[P] = \frac{(\Delta ABS) - \alpha_s [S]_{t0}}{\alpha_p - \alpha_s} \quad (1)$$

with α_s and α_p representing absorption coefficients of enzyme substrate and product, respectively. ΔABS was calculated as the difference of the median absorbances measured in the reaction and

the reference wells. Finally, potential enzyme activity was expressed as $\Delta P\ DW^{-1}\ h^{-1}$.

2.7. Data analysis & statistics

Results were analysed using R (R Core Team, 2012) and the R packages *nlme* (Pinheiro et al., 2012) for linear-mixed effect modelling and *car* (Fox and Weisberg, 2011) for type II sums of squares. Treatment effects on porewater nutrient concentrations were tested using the non-parametric Wilcoxon Rank Sum test due to the strong heteroscedasticity. The other data were fitted to linear mixed-effects models with nutrient treatment or zone as fixed factor and with transect and sampling location as random factors where appropriate. Homoscedasticity was confirmed by Levene's test and normality was checked using Shapiro's test. In the enzyme analysis, outliers were identified as being more than two times the interquartile range from the first or the third quartiles resulting in the removal of 2% of the data points. Treatment effects for random models were tested using Walds χ^2 test with type II sums of squares.

3. Results

3.1. Soil and mangrove characteristics

Fringe and dwarf zones differed with respect to porewater nutrient and DOC concentrations (Table 2): In the dwarf zone, DOC and NH_4^+ concentrations were higher than in the fringe zone, while the fringe zone contained more PO_4^{3-}. Soil organic nitrogen (SON) content of the porewater was slightly lower in the fringe zone, while soil organic carbon (SOC) content did not differ between the two zones.

At the time of sampling, nutrient levels in the porewater clearly reflected the fertilisation treatments in the dwarf zone, while this was much less evident in the fringe zone (Table 2). The N fertilisation led to a higher NH_4^+ porewater concentration in the dwarf, but not in the fringe zone. The P fertilisation increased porewater PO_4^{3-} concentrations in both zones, but this increase was six times higher in the dwarf zone as compared to the fringe zone.

Phosphorus fertilisation significantly increased tree height in the dwarf zone, but not in the fringe zone, while N fertilisation did not affect tree height in either of the zones (Fig. 1a, Table 3). The same was true for relative surface level, which in the dwarf zone was 11 cm higher in the P treatment as compared to the control (Fig. 1b, Table 3) and for concentrations of soluble phenolic compounds, which were also higher in the P-fertilised dwarf plots (Fig. 1c, Table 3). Insoluble phenolic compounds showed a different pattern: P-fertilised sites had higher concentrations of insoluble phenolic compounds in both the dwarf and fringe sites as compared to the control treatment, but there was again no effect of N fertilisation (Fig. 1d, Table 3).

Table 2
Measured nutrient and carbon contents in porewater and soil from nitrogen- (N) and phosphorus- (P) fertilised and unfertilised (Control) plots in *Rhizophora mangle*-dominated sites in the fringe and dwarf zones of Twin Cays, Belize. Symbols denote significance levels (*** $p<0.001$, ** $p<0.01$, * $p<0.05$, $^{\cdot}$ $p<0.1$) of zonation effects under Control, and treatment effect under N and P. Significance levels result from either the non-parametric Wilcoxon Rank Sum test (W) for mineral nutrients or Wald's χ^2 ANOVA analysis of a mixed effects model with transect as a random factor and N and P or Zone as fixed factors for the organic nutrients.

	Dwarf			Fringe			
	Control	N	P	Control	N	P	
NH_4^+	0.03^{**}	0.86^{***}	0.02	0.01^{**}	$0.06^{\cdot}$	n.d.	mmol l^{-1}
PO_4^{3-}	n.d.**	n.d.	0.30^{***}	0.002^{**}	n.d.	0.05^{***}	mmol l^{-1}
NO_3^-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	mmol l^{-1}
SOC	336^{***}	$304^{\cdot}$	335	359^{***}	379	344	mg Cg soil DW^{-1}
SON	14^{*}	13	14	16^{*}	15	12^{*}	mg Cg soil DW^{-1}
DOC	3.3^{**}	2.8	4.2	1.9^{**}	2.8	1.8	mmol l^{-1}
DON	n.d.*	n.d.	0.02	0.04^{*}	n.d	0.05	mmol l^{-1}

SOC: soil organic carbon, DOC: dissolved organic carbon, SON: soil organic nitrogen, DON: dissolved organic nitrogen, n.d = non-detectable.

3.2. Soil microbial characteristics

Field fertilisation modified both microbial biomass and elemental stoichiometry (Fig. 2, Table 3). In the control, microbial biomass was much lower in the dwarf zone as compared to the fringe zone. Also, the relative C content of microbial biomass was lower in the dwarf zone as reflected in the lower C:N and C:P ratios. Microbial N:P did not differ between the two zones. Compared to the average ratios for marine microbial biomass as given by Fagerbakke et al. (1996), the relative N content was low in both zones, while the relative content of C is somewhat below average for the dwarf, and above average for the fringe zones.

In the dwarf zone, N fertilisation decreased microbial biomass, C:N, and C:P, while N:P remained unaffected. P fertilisation quintupled microbial biomass, while it increased C:N marginally. C:P and N:P were not significantly different from the control treatment.

Within the fringe zone, N and P fertilisation had similar effects on microbial biomass with lower values in both the N and the P treatments. Mean C:N was not significantly different between control and treatments, while C:P was significantly lower in both the N and the P treatments. In both zones, microbial N:P remained unaffected by either fertilisation treatment.

3.3. Enzyme activities in the field

Potential activities of hydrolytic enzymes involved in N, P, and C acquisition as well as potential activities of the oxidative POX enzyme were measured in fresh soil collected from both the fringe and the dwarf zone (Fig. 3 and Table 4). Between dwarf and fringe plots only the potential AP activity differed significantly, with a somewhat higher activity in the control dwarf plot. N-fertilisation led to a higher potential AP activity in the fringe, but not in the dwarf zone. P-fertilisation caused an increased potential GAP and a decreased potential POX activity in both the dwarf and the fringe zone, while it increased potential activities of CBH and LAP in the fringe zone only. No POD activities are shown, because no POD activity was detectable at either of the sites.

3.4. Enzyme activities in the short-term incubation experiment

Responses of potential enzyme activities to short-term nutrient amendments in laboratory incubations of dwarf control soils were generally much stronger than those observed for long-term nutrient fertilisation in the field (Fig. 4, Table 5). Glucose amendment increased potential activities of BG, LAP, and AP. Without glucose, N amendment had the strongest effect with significant increases in potential BG, LAP and AP activities as compared to the control. P amendment only increased potential LAP activity. Within the glucose treatment, potential BG, NAG, LAP and AP activities were stimulated by N addition. The amendment of P stimulated potential BG activity while it decreased potential AP activity. Both with and without glucose, combined N and P amendment lead to lower potential activities of LAP and AP as compared to amendments of N without P.

CBH and GAP were not significantly affected by any of the nutrient treatments. This result is in contrast to the observed responses to P enrichment in the long-term fertilisation experiment

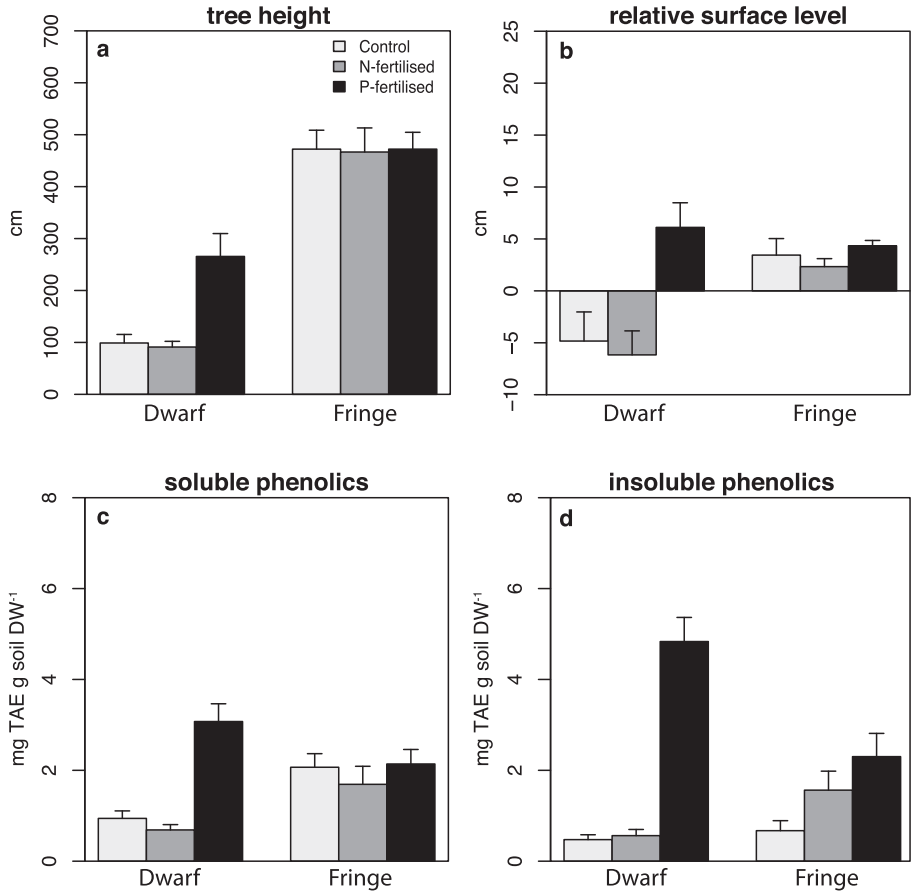

Fig. 1. Site characteristics in *Rhizophora mangle*-dominated sites at Twin Cays, Belize. The inner 'dwarf' zone showed stunted growth of *Rhizophora* trees, while the outer 'fringe' zone showed normal growth. Sites were fertilised with nitrogen (grey bars) or phosphorus (black bars). Relative surface level is defined against the Mean Lower Low Water (MLLW) level. Phenolic concentrations are expressed in tannic acid equivalents (TAE).

in the dwarf zone. Conversely, the high sensitivity of AP to short-term N and glucose enrichment contrasts with the relatively weak responses observed in the long-term treatments.

4. Discussion

4.1. Elemental stoichiometry

We hypothesised that microbial decomposers would be N-limited in Twin Cays due to the protein binding capacity of mangrove-derived tannin compounds, even in the dwarf zone where plant growth is P-limited. The elemental stoichiometry of the microbial community in the field supports this hypothesis. Without fertilisation, microbial N:P ratios were below the average 1:10, and C:N ratios were three to four times above the average of 1:5 for aquatic microorganisms (Fagerbakke et al., 1996; Cleveland and Liptzin, 2007). In the dwarf zone, N fertilisation restored the C:N ratios to average global values, but the low N:P ratios decreased even further. This indicates that microorganisms were able to accumulate substantial amounts of P, even in the sites where P strongly limits primary production.

Nevertheless, P enrichment quintupled microbial biomass in the dwarf zone, while N amendment had no effect. This is seemingly contradictory to the stoichiometric response to fertilisation. Thus, the soil microbial community appears to also be P-limited like the primary producers, or microbial growth is indirectly stimulated by higher primary production, for instance through increased rhizodeposition and litter production. Available evidence suggests the latter. In the dwarf zone, P fertilisation increased primary production (Feller et al., 2002) and C input to the soil (McKee et al., 2007). At the same time, the microbial C:P ratio increased in our plots. In other words, relative microbial P concentrations decreased despite increased availability of P. The observed increase in microbial biomass upon P enrichment in the dwarf zone thus appears to be the result of increased C input, rather than increased P availability.

4.2. Enzyme activities in incubation experiments

Enzyme activity responses to amendments of nutrients and carbon were measured in short-term laboratory incubations to isolate the direct responses of soil matrix enrichment from plant-mediated responses. Two types of responses were observed in the incubation experiment: i) A change in overall exoenzyme activity, without an apparent shift in relative enzyme activities. We interpret this as increased overall enzyme production due to the alleviation of a general limitation to microbial activities; ii) A change in relative enzyme activity. Relative activities of C- and nutrient-acquiring exoenzymes are often utilised as an indicator for the nature of the nutrient limitations for microbial decomposers, under the assumption that microbial communities optimise their allocation of resources to maximise their productivity (Sinsabaugh, 1994; Sinsabaugh and Moorhead, 1994; Sinsabaugh et al., 2010).

Table 3
ANOVA-table with Walds χ^2 values and significances of fertilisation (N, P) and zonation (Zone) in a mixed effects model of site, soil and microbial properties in fringe and dwarf *Rhizophora mangle* -dominated sites with transect as a random factor. Symbols denote significance levels (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, $\cdot$ $p < 0.1$).

		Tree height	Soil elevation	Soluble phenolics	Insoluble phenolics	Microbial			
						Biomass	C:N	C:P	N:P
Zone		182.9***	8.11	42.10***	0.77	71.86***	8.60**	14.75***	0.73
Dwarf	N	0.05	0.37	1.43	0.11	7.48**	8.52**	5.02*	1.66
	P	25.28***	24.89***	34.42***	112.4***	53.20***	3.29 $\cdot$	1.14	0.11
Fringe	N	0.12	0.65	0.80	2.01	9.46**	3.21 $\cdot$	6.10*	0.08
	P	0.00	0.42	0.06	9.15**	7.37**	3.40 $\cdot$	12.80***	0.26

Single amendments of N, P, and labile organic C all yielded similar increases in overall hydrolytic enzyme activity, without markedly changing relative activities (Fig. 4, upper half). From this, we conclude that the microorganisms producing these exoenzymes are co-limited by each of those elements. This result confirms the observation that Liebig's law of the minimum does not apply to microbial decomposers (Kaspari et al., 2008) possibly due to the large number of simultaneously active species (Danger et al., 2008). Amendments of either N or P did not have an effect, but in the presence of C, their effect was highly synergistic (i.e. with a positive interaction). From this, we conclude that labile organic C was the most limiting factor for exoenzyme production. Within the C-amended treatments, the effect of N on enzyme activity was considerably larger than the effect of P. This is in line with the finding from stoichiometry that N is more limiting to microbial activity than P. In contrast to the findings of Shackle et al. (2000), we observed that enrichment with labile C increased activity of exoenzymes related to nutrient acquisition, as also predicted by the microbial resource optimisation model (Sinsabaugh and Moorhead, 1994).

Stimulation of specific exoenzymes in response to short-term amendments was also observed, although not all responses were easy to interpret. Upon N amendment, potential activity of the P-acquiring AP enzyme increased, which matches expectations based on optimal resource allocation. Activity of the N-harvesting enzyme LAP could however not be explained using this principle, as its

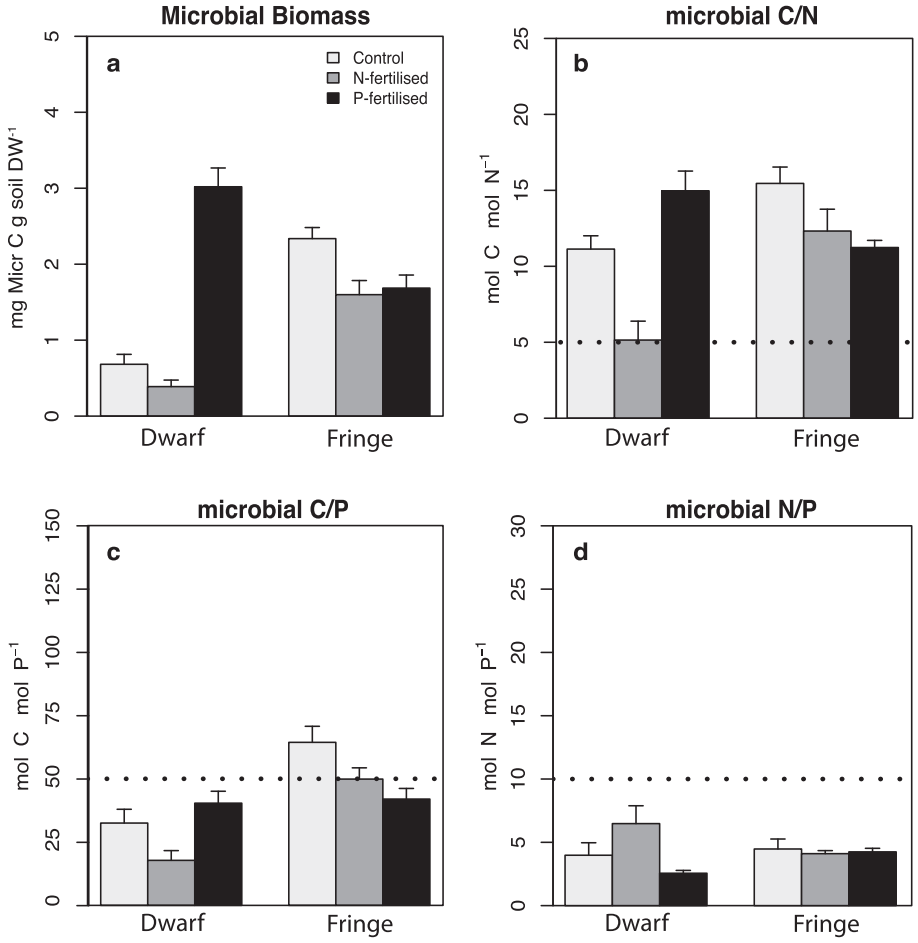

Fig. 2. Soil microbial characteristics in *Rhizophora mangle*-dominated sites at Twin Cays, Belize. Two types of *Rhizophora mangle*-dominated sites are distinguished: The inner 'dwarf' zone with stunted growth of *Rhizophora mangle* trees, and the outer 'fringe' zone where *Rhizophora mangle* trees have a much taller growth form. Plots in each zone were left unfertilised (light-grey bars), nitrogen-fertilised (dark-grey bars) and phosphorus-fertilised (black bars). The horizontal lines indicate average values for marine bacteria as given by Fagerbakke et al. (1996).

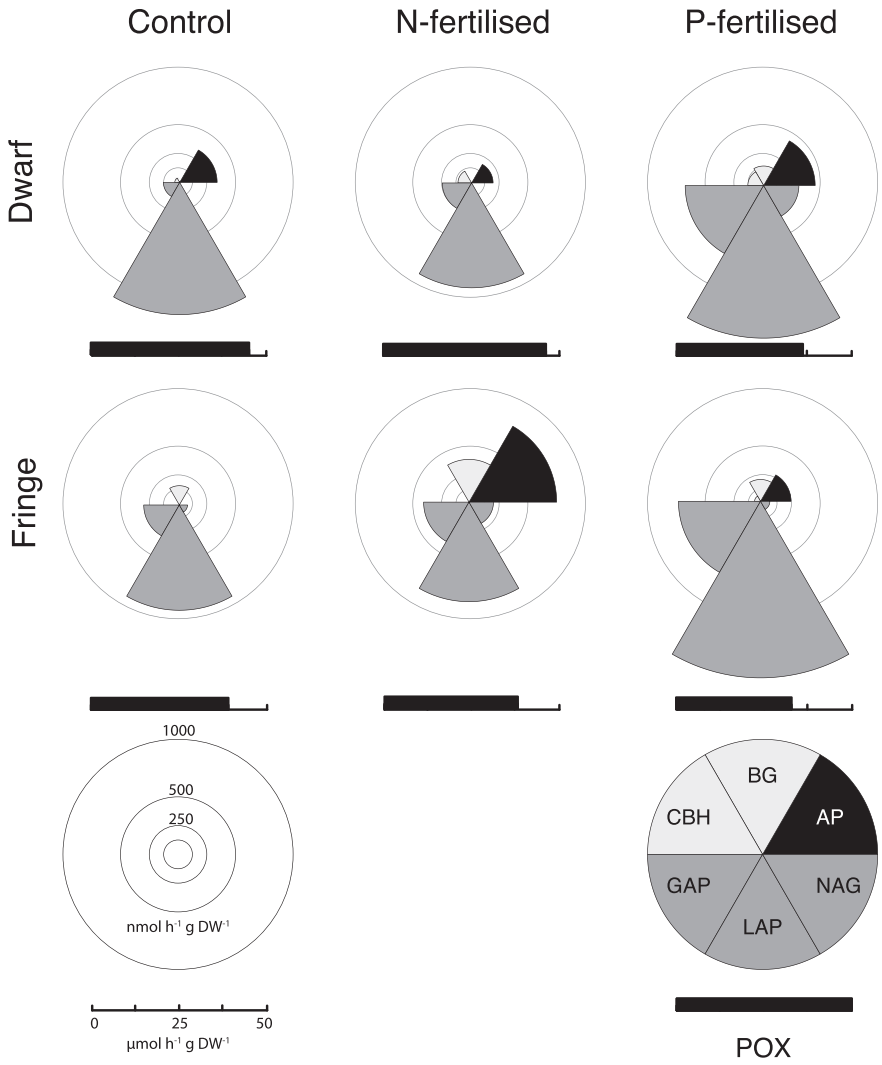

Fig. 3. Potential activity of oxidative (bars) and hydrolytic microbial exoenzymes (segments) involved in acquiring carbon (light-grey), nitrogen (dark-grey) and phosphorus (black) in soils dominated by *Rhizophora mangle* with a dwarf (top series) or fringe growth form (middle series). Sites from both zones were subject to long term fertilisation with either nitrogen (N) or phosphorus (P). Hydrolytic enzymes are plotted as circle segments with radius indicating potential activity (AP = phosphatase, NAG = β-N-acetylglucosaminidase, LAP = leucine aminopeptidase, GAP = glycine aminopeptidase, CBH = cellobiohydrolase, BG = β-1,4-glucosidase). Potential POX (*phenol oxidase*) activity is plotted on the bars below the circles.

activity increased with all amendments, including N amendment. Similarly, potential NAG activity increased upon N addition if also glucose was given. Although LAP and NAG are generally considered to be only involved in N harvesting (Sinsabaugh et al., 2009), the end products of both enzymes, leucine ($C_6H_{13}NO_2$) and N-acetyl-glucosamine ($C_8H_{15}NO_6$) respectively, contain considerably more C than N. This means that LAP and NAG activities contribute more to energy and C uptake than to N uptake, which may explain the unexpected increase in activity even when enough mineral N was available. The same is true for the end product of GAP (galanine – $C_2H_5NO_2$), but in our study, its activity did not respond to any of the given amendments.

4.3. Enzyme activity in the field fertilisation experiment

In the field experiment, responses of enzyme activities to nutrient amendments were much weaker as compared to the laboratory incubations, but they roughly followed the same direction. In contrast to the short-term response, potential LAP, BG and NAG activities were not significantly stimulated by N amendment. The increased potential activities of AP in the N fertilised dwarf and fringe zones indicate that P became the limiting nutrient, which was not the case without amendment. Conversely, P fertilisation increased the potential activity of the N-acquiring enzymes LAP and GAP, indicating that N had become limiting, upon amendment. The latter is not in accordance with the elemental stoichiometry of the microbial biomass, which suggests that N is also limiting in the control sites. Similar to the short-term incubations, the largest activity increase of N-acquiring enzymes was observed for LAP. This strengthens the suspicion that this enzyme is less specific than previously thought, although the increased activity might also be explained by a more acute N limitation in the rhizosphere due to increased plant production. The potential activity of the oxidative POX enzyme decreased after P fertilisation, while N did not have an effect. This is in contrast to other studies, which report a decline of POX activity upon N amendment (Carreiro et al., 2000; Saiya-Cork et al., 2002; DeForest et al., 2004) and a neutral effect of P amendment (Wright and Reddy, 2001).

Despite the substantially higher microbial biomass in the P fertilisation treatment in the dwarf zone, the potential enzyme

Table 4
ANOVA table with Walds χ^2 test values of a mixed effects model of the potential enzymatic activity in *Rhizophora mangle*-dominated sites in the dwarf and fringe zone with transect as a random factor and fixed factors printed in boldface. Nutrient effects were separately tested for the dwarf and the fringe zone, the effect of zone was tested on the control treatment only. Symbols denote significance levels (*** $p < 0.001$,** $p < 0.01$,* $p < 0.05$, $^{\cdot}$ $p < 0.1$).

		BG	CBH	NAG	LAP	GAP	AP	POX
Zone		1.86	1.51	1.30	2.11	2.59	7.81**	3.79 $^{\cdot}$
Dwarf	N	0.00	1.65	2.38	1.62	0.43	0.12	0.43
	P	3.81 $^{\cdot}$	2.38	1.82	0.62	8.63**	0.01	6.99**
Fringe	N	3.67 $^{\cdot}$	0.66	0.98	0.15	0.23	10.5**	0.11
	P	0.13	4.63*	0.02	23.5***	8.60**	5.36*	5.89*

AP = phosphatase, NAG = β-N-acetylglucosaminidase, LAP = leucine aminopeptidase, GAP = glycine aminopeptidase, CBH = cellobiohydrolase, BG = β-1,4-glucosidase, POX = phenol oxidase.

Table 5
ANOVA table with Walds χ^2 test values of a mixed effects model of the potential enzymatic activity in stunted 'dwarf' *Rhizophora mangle*-dominated sites with sample location as a random factor, and amendments as fixed factors (N = nitrogen, P = phosphorus, C = glucose). Nutrient effects were separately tested in incubations with and without C amendment, the effect of C amendment was tested in unfertilised incubations only. Symbols denote significance levels (*** $p < 0.001$,** $p < 0.01$,* $p < 0.05$, $^{\cdot}$ $p < 0.1$).

		BG	CBH	NAG	LAP	GAP	AP
C		5.17*	0.03	1.16	7.03**	3.06 $^{\cdot}$	5.66*
−C	N	7.96**	1.20	3.23$^{\cdot}$	7.30**	0.03	4.87*
	P	0.01	1.15	3.66$^{\cdot}$	7.08**	0.10	0.58
	N*P	0.45	0.37	0.61	9.02**	0.15	4.41*
+C	N	7.27**	2.38	9.19**	4.59*	0.01	19.4***
	P	22.2***	0.01	0.70	0.06	0.89	6.06*
	N*P	2.11	1.57	2.59	11.2***	0.51	13.4***

AP = phosphatase, NAG = β-N-acetylglucosaminidase, LAP = leucine aminopeptidase, GAP = glycine aminopeptidase, CBH = cellobiohydrolase, BG = β-1,4-glucosidase.

activities did not increase, indicating that the enzyme production per unit microbial biomass decreased in these treatments. This is consistent with the hypothesis that tannins specifically inhibit microorganisms capable of producing-exoenzymes. P enrichment consistently decreased POX activity and therefore inhibits decomposition of recalcitrant organic matter. Thus, as an indirect effect of P amendment, microbial production and turnover of freshly produced labile C compounds increased, as indicated by higher microbial biomass. This higher C turnover and higher microbial biomass did not lead to a positive priming effect on the breakdown of recalcitrant organic matter, as shown by the similar potential activities of hydrolytic enzymes and the lower POX activity in comparison to the non-fertilised plots.

5. Conclusions

Potential exoenzyme activities have been used to predict microbial decomposer nutrient limitation (Sinsabaugh et al., 1993). We showed that in mangroves responses of potential exoenzyme activities upon short-term nutrient amendment proved to be similar to long term responses. Short-term incubations are therefore a useful tool to predict microbial enzyme responses, without the confounding effects of plant responses to nutrient enrichment. Not all responses in exoenzyme activities could however be directly linked to microbial stoichiometry and nutrient limitation. The end

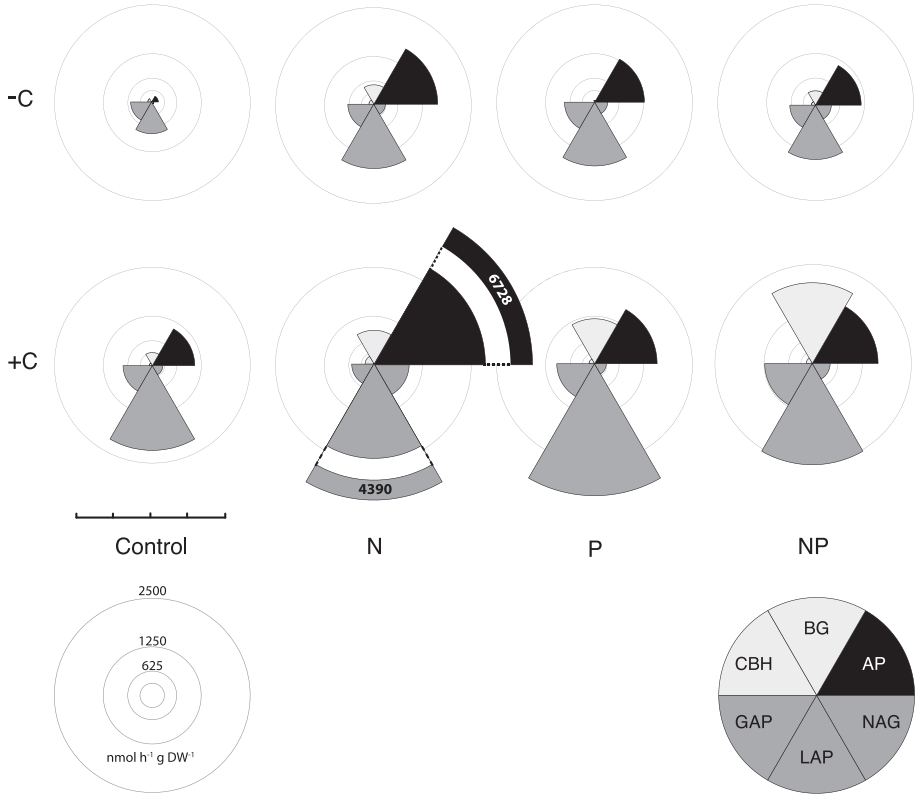

Fig. 4. Potential activity of hydrolytic microbial exoenzymes (segments) involved in acquiring carbon (light-grey), nitrogen (dark-grey) and phosphorus (black) in soils dominated by stunted 'dwarf' *Rhizophora mangle* after four days of incubation with amendment of various combinations of ammonium (N), phosphate (P) and glucose (C). Hydrolytic exoenzymes are plotted as circle segments with radius indicating potential activity (AP = phosphatase, NAG = β-N-acetylglucosaminidase, LAP = leucine aminopeptidase, GAP = glycine aminopeptidase, CBH = cellobiohydrolase, BG = β-1,4-glucosidase).

products of the N-harvesting enzymes GAP, LAP and NAG not only contain N but also significant amounts of C and could therefore also serve as sources of C and energy. A similar finding was reported by Steenbergh et al. (2011) for the 'P-harvesting' enzyme AP, which was shown to relieve microbial C limitation in a marine soil. The presumed distinction in 'P-harvesting', 'N-harvesting' and 'C-harvesting' enzymes is therefore not as clear-cut as often suggested (in e.g. Sinsabaugh et al., 2009) and needs to be reconsidered.

The microbial stoichiometry was consistent with the hypothesised N limitation in the mangrove soils studied. The expected increase in peat decay due to N addition did not occur, as enzyme production appears to be mostly limited by energy availability. Overall, the effects of P enrichment were much larger than that of N, despite the fact that microbial decomposers are N-limited. In the P-limited dwarf site, P increased the turnover of labile C compounds, as indicated by the threefold increase in microbial biomass, but decreased the turnover of recalcitrant compounds as indicated by the lower POX activity. This result has been reported for inorganic N (e.g. Sinsabaugh, 2010; Hobbie et al., 2012), but not for P enrichment.

Our results suggest that the observed responses to nutrient amendments depend on which nutrient limits primary production. The more frequent occurrence of N-limited ecosystems then would lead to a bias towards observation of strong N-effects. Thus explicit comparison of nutrient enrichment responses in P- versus N-limited systems represents a pressing research priority.

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