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Nitrogen deposition may increase litter accumulative CO_2 release in a subtropical estuarine marsh

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Microbial evolution-mediated CO_2 from litter has aroused widespread concern, and knowing the factors controlling litter-derived CO_2 is important when considering the effects of accumulative CO_2 release from litter on the global greenhouse. We conducted a short-term N addition (6, 16, and 24 g N m^{-2} yr^{-1}) experiment in *Cyperus malaccensis* var. *brevifolius* (shichito matgrass) litter decomosition. Phospholipid fatty acid (PLFA) method and enzyme method were used to analysis litter microbial community composition and enzymatic activity. During a 220-day decomposition period, there was little effect of the N amendments on litter CO_2 evolution rates (9.97–307.54 μg C g^{-1} h^{-1}) with a notable exception regarding the increase of the high-N treatment at day 20. The accumulative CO_2 release significantly increased after N addition in the medium and late phases. The facilitation effect on accumulative CO_2 release by N amendments was more and more obvious over the decomposition time, especially for the low- and intermediate-N treatments. At the end of our experiment, compared with the control treatment, accumulative CO_2 release increased 69.75%, 76.62%, and 39.93% for low-, intermediate-, and high-N treatments, respectively. These observations highlight that N deposition could cause high losses of litter C as CO_2.

KEYWORDS

litter-derived CO_2, litter decomposition, nitrogen addition, estuarine marsh, accumulative CO_2 release

Introduction

Humans continue to transform the global nitrogen (N) cycle at a record pace, and serious N pollution could generate unacceptable environmental change (Galloway et al., 2008; Rockström et al., 2009). Significant fractions of this anthropogenic N enter coastal estuaries, and contributed to numerous eco-environmental problems, such as widespread eutrophication and accelerating greenhouse gasses emission (Simas and Ferreira, 2007; Lin et al., 2017; Lin and Lin, 2022). Previous studies have revealed that N deposition significantly promoted plant height and biomass, changed litter matrix, inhibited litter decomposition, and affected soil carbon (C) storage in the estuarine wetlands (Guan et al., 2019; Tao et al., 2019). To sequester more C in soil, we need to consider how to divert more litter into humus (Prescott, 2010), and to decrease fraction of litter C released as CO_2 to the atmosphere.

Despite growing research interest, uncertainties remain on the response of litter decomposition to the N amendments due to the different ecosystems, species, decomposition stages, and N thresholds (Knorr et al., 2005; Xu et al., 2016; Zhang et al., 2018; Ochoa-Hueso et al., 2019). Previous studies have demonstrated that N amendments may promote (Gerdol et al., 2007), inhibit (Tao et al., 2019) or no significant effect (Yu et al., 2019) on the litter decomposition rate in wetland. For decades, litter-derived CO_2 has aroused widespread concerns (Kuehn et al., 2000; Chambers et al., 2001; Kuehn et al., 2004; Zhang et al., 2014b; Hall et al., 2017; Mao et al., 2021). The fraction of litter C released as CO_2 to the atmosphere is nearly 30% of the total C (TC) (Rubino et al., 2010), that represents a substantial pathway of C input to the atmosphere (Day et al., 2018). Li et al. (2015) suggested that the combination of litter and N addition increased CO_2 release although N fertilization alone significantly inhibited CO_2 release rates. Simulated CO_2 emissions from soil fertilized with litter averaged across years were approximately 0.8 times higher than soil fertilized with NH_4NO_3 (Yang et al., 2019). Magill and Aber (2000) suggested that N inputs appear to affect the quantity of litter C consumed or released by increasing respiration (as measured by weight loss), rather than increasing litter-derived DOC release into the soil solution. In freshwater marshes, the N addition could significantly increase CO_2 emission (Hu et al., 2019a), however, the response of litter-derived CO_2 to N amendments remain poorly understood (Li et al., 2015). To better understand the C cycle in estuarine marshes, the fate of litter-derived CO_2 and its driving mechanism need to be identified.

Here, we asked a simple question: Does N deposition affect litter-derived CO_2 evolution rates and accumulative CO_2 release during litter decomposition? According to the results of previous research concerning an increase in litter-derived respiration (Magill and Aber, 2000) and an increase in litter input (Liu and Greaver, 2010; You et al., 2017), and a decrease in litter decomposition (Tu et al., 2011; Xu et al., 2016) after N fertilization, we hypothesized that, (1) N addition could increase litter-derived CO_2 evolution rates; and (2) consequently increase accumulative CO_2 release due to large amounts of remaining litter residue.

Materials and methods

Site description

Our study was conducted in a freshwater marsh, namely Tajiaozhou (25°56′48″ N; 119°22′1″ E), in the Min River Estuary (Figure 1). Located in the transition zone between the middle and southern subtropical zones, this area is exposed to an East Asian monsoon climate with annual mean temperature and precipitation of 19.7°C and 1200–1740 mm, respectively (Luo et al., 2019). Background N deposition of southeast China was

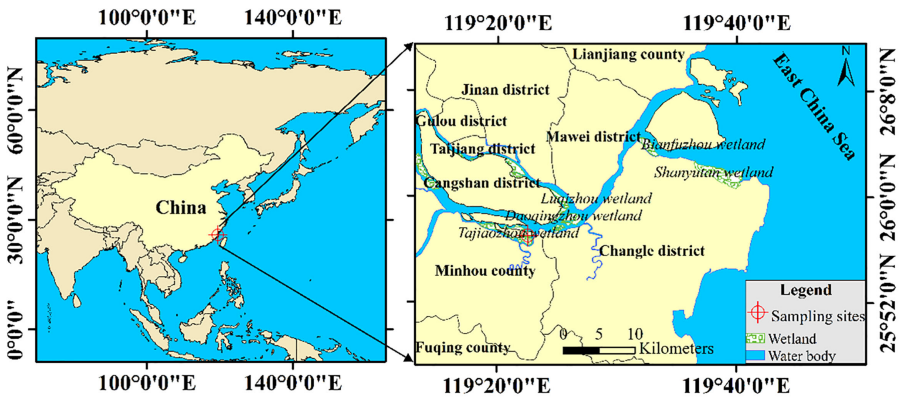

FIGURE 1
Study sites for the leaf-bag decomposition experiments.

estimated as 41.7 kg N ha^{-1} a^{-1} (Xu et al., 2015). This marsh experiences a semi-diurnal tide, and the inundation frequency at the measurement sites generally varied between 0 and 31.80%. The sediment is neutral or faintly acidic (~pH 6.11). *Cyperus malaccensis* var. *brevifolius* (shichito matgrass), a type of grass-like perennial, is a typical native species in this marsh.

Experimental design and treatments

We conducted a short-term N addition experiment during 220 days. To evaluate the effects of future elevated N-saturated conditions on litter-derived CO_2, the N levels applied in this study were in line with background N loading. N eutrophication symptoms in rivers discharging to oceans are mainly driven by nitrate (NO_3^-) in subtropical and temperate estuarine areas (Meybeck and Ragu, 2012), and thus additional reactive N was applied as $NaNO_3$ in this experiment. Three N treatments were designed with doses of 6 g N m^{-2} yr^{-1} (low N), 16 g N m^{-2} yr^{-1} (intermediate N), and 24 g N m^{-2} yr^{-1} (high N), and a control treatment was set up with no N added.

Two experimental blocks (I and II) were established at the sites with uniform vegetation, soil, and hydrological characteristics, which remained completely exposed during low or neap tide. In each block, there were four 0.6 m × 0.6 m plots (decomposition boxes, polyvinyl chloride) with 1-m buffers that were assigned to receive N treatments, giving a total of 12 treatment plots in each block. Additional N was added to *in situ* tidewaters (1 L) and supplied at neap tide twice a month, whereas control plots received additional and equivalent tidewater only. In block II, 12 porewater sample collectors were placed in each plot. Litter from block I was analyzed to determine the 2elemental composition of litter remains and litter properties. Litter from block II was used to determine CO_2 evolution rates and phospholipid fatty acid (PLFA) content of litter.

Standing dead litter of shichito matgrass stems were collected to serve as test material in March 2017. To unify the microbial levels of initial decomposition material, the collected litter was first washed with filtered stream water and then with deionized water in the laboratory. Litter material was cut into 5-cm long pieces, and then air-dried at approximately 18°C (mean daily temperature) for several weeks. A subsample of this air-dried material was weighed, oven-dried at 60°C, and reweighed to calculate the moisture correction factor for calculating the initial mass of the air-dried litter. Litter decay processes were investigated using the experimental leaf-bag technique (pre-dried litter enclosed in litterbags). For this, an aliquot of approximately 15 g (dry weight) was transferred to each litterbag (20 cm × 15 cm, prepared using 0.2-mm fiberglass mesh). Total 192 litterbags were placed in the experimental field (i.e., 2 blocks × 8 sampling time points × 4 treatments × 3 replicates). These litter-bags were tied to polypropylene canes that were buried in the ground to prevent their displacement by the tide (Zhang et al., 2014a). Based on the findings of Hu et al. (2019b) regarding shichito matgrass litter decay, and the litter decay phases described by Valiela et al. (1985), we set three retrieval phases. Litterbags were retrieved at 10-day intervals (early phase: days 10, 20, and 30), 30-day intervals (medium phase: days 60, 90, and 120), and 50-day intervals (late phase: days 170 and 220). In total, 24 litterbags were collected at each sampling interval. Litterbags were carefully retrieved and placed in sealed plastic bags in a portable cooler, and then immediately transported to the laboratory.

Litter properties

Litter remaining in the bags was washed gently and weighed after oven-drying to a consistent mass. The dried litter was then ground and passed through a 100-mesh (0.149-mm) sieve, and litter TC, total nitrogen (TN), and total sulfur (TS) concentrations were determined using a Vario EL Elemental Analyzer (Elementar Vario EL, Frankfurt, Germany). Litter-derived DOC was extracted from 0.5 g dry litter as described by Uselman et al. (2012), and analyzed using a total organic carbon analyzer (Shimadzu TOC-V_{CPH}, Kyoto, Japan)

Environmental parameters

The *in-situ* sediment pH was measured using an IQ150 instrument (IQ Scientific Instruments, Carlsbad, CA, USA), and electrical conductivity (EC) was measured using a 2265FS EC meter (Spectrum Technologies Inc., Aurora, CO, USA). TC and TN concentrations of the top 2 cm of sediment were determined using a Vario MAX CN element analyzer (Elementar). Porewater from the sample collectors was transferred into 50-mL plastic cups (polypropylene, acid washed) and filtered (0.45-μm membrane filters), and then analyzed for TN and dissolved inorganic N (DIN = $NH_4^+ + NO_3^- + NO_2^-$) using a continuous flow-injection analyzer (SKALAR San^{++}, Breda, the Netherlands) (Huang et al., 2021). The crab hole density, plant height, and plant density were measured within the plots (0.6 m × 0.6 m).

Litter CO_2 evolution rate assay

The assay we used for determining litter CO_2 evolution rates has been described previously (Zhang et al., 2014b). In brief, sterile tweezers were used to carefully remove visible debris and soil from fresh litter. Then the fresh litter samples (4 g fresh weight) were placed into sterile glass incubation jars (250 mL) containing sterile filter paper. An additional three jars containing a filter paper without plant material were used as controls. All jars were wetted until saturation with sterile

deionized water, placed in an incubator, pre-incubated for 2 h, and then sealed and incubated at 20°C (annual mean temperature). Gas samples were collected at 0, 4, and 8 h after the chamber was closed using a 20-mL syringe equipped with a three-way stopcock and were stored in gas sampling bags (Dalian Delin Gas Packing Co., Ltd., Dalian, China). The CO_2 concentrations were determined within 12 h of sampling using a gas chromatograph (GC-2010, Shimadzu).

Litter phospholipid fatty acid and enzyme assay

Litter PLFAs were extracted from 1 g of fresh litter according to Hassett and Zak (2005) and Rejmánková and Houdková (2006). Microbial biomass was calculated by summing total PLFAs (C_{14}–C_{20}), and PLFAs specific to fungi (18:2ω6,9c), gram-positive bacteria (i14:0, a16:0, i15:0, a15:0, i16:0, i17:0, and a17:0), and gram-negative bacteria (16:1ω7c, cy17:0, 18:1ω7c and cy19:0) were summarized separately (Feng and Simpson, 2009).

Fresh litter samples were assayed following published protocols for β-glucosidase and cellobiohydrolase using methyumbelliferyl-β-glucoside and methyumbelliferyl-cellobioside as substrates, respectively (Saiya-Cork et al., 2002; Sinsabaugh et al., 2005). Fluorescence was measured using a Multiscan Spectrum (Synergy H4, USA) calibrated for excitation at 365 nm, and with emissions set at 460 nm. All enzymatic activities were calculated as nmol h^{-1} g^{-1} OM.

Statistical calculation

We focused on CO_2 evolution from the residual mass of litter; thus, the litter residual mass (R, %) was calculated as follows:

$$R = W_t / W_0 \times 100\%$$

where W_0 (g) is the original dry mass, and W_t (g) is the dry mass at a time "t", and t (d) is decomposition time in days.

CO_2 evolution rates were calculated according to previous research as follows (Zhang et al., 2014b):

$$y_T = \frac{(dc/dt) \times (M \times V \times P \times T_0)}{(m \times V_0 \times P_0 \times T)}$$

where y_T is the CO_2 evolution rate (μg g^{-1} dry weight h^{-1}); dc/dt is the slope of the linear regression for gas concentration gradient through time (μL L^{-1} C h^{-1}); M is the atomic mass of C (12); V is the volume of the jar (L); P is the atmospheric pressure (MPa); m is the litter mass in jars (g); T is the absolute temperature during sampling (K); and V_0, P_0, and T_0 are the gas mole volume (L), atmospheric pressure (MPa), and absolute temperature (K) under standard conditions, respectively.

Accumulative CO_2 release (per initial dry weight per m^2) was calculated by integrating the area under the curve for all dates, based on the assumption that rates of CO_2 loss scaled linearly between time points (Jacobs et al., 2018) as follows:

$$y_t = x_t \times M_t \times 24 \times 10^{-3}$$

$$f(y) = ky + b$$

$$S_i = \int_t^{t+1} f(y)\ dt$$

$$AR_i = M_0 \times 10^{-3} \times \sum_{i=1}^{n} S_i$$

where AR_i is the accumulative CO_2 release per initial dry weight per m^2 at time i (g m^{-2}); x_t and y_T are the CO_2 evolution rates per hour (μg g^{-1} h^{-1}) and per day (mg), respectively; M_t is the residue mass of litter at time "t" (g); $f(y)$ is the accumulative CO_2 release during decomposition; S_i is accumulative CO_2 release at the interval between sampling times (mg g^{-1}); M_0 is the initial litter mass per m^2 (g m^{-2}), and the average yield of litter at control, low-N, intermediate-N, high-N treatments were 558.38 g m^{-2}, 1000.18 g m^{-2}, 723.67 g m^{-2}, and 633.52 g m^{-2}, respectively (unpublished data); n is the number of sampling time.

Data analysis

All datasets were tested for normality by the Shapiro-Wilk test, and homogeneity of variance by the Brown-Forsythe test. If these assumptions were not met, then the raw data were log transformed before further statistical analysis. One-way analysis of variance (ANOVA) and repeated measure analysis of covariance (ANCOVA) were used to assess differences among samples (SPSS 19.0, IBM, Armonk, NY, USA) with a significance level of $p < 0.05$.

Redundancy analysis (RDA) was used to partition the variation in CO_2 evolution rates and accumulative CO_2 release explained by environmental parameters, microbial biomass, and litter quality (Wang et al., 2015), and to interpret the extent and direction of compositional changes; these analyses were performed using Canoco 5.0.

Structural equation modeling (SEM) was performed to analyze the causal mechanisms underlying the response of litter accumulative CO_2 release to N addition using SPSS Amos 21.0 (IBM). The best-fit SEM was derived by maximum likelihood and the model fit was determined using chi-square tests (χ^2), p-values, goodness-of-fit index (GFI), root mean square errors of approximation (RMSEA), and Akaike information criteria (Zhu et al., 2018). The litter CO_2 evolution rates and accumulative release were generated using Origin 9.3 (OriginLab Corporation, Northampton, MA, USA),

and a conceptual framework was created using Microsoft Office Visio 2016 (Microsoft Corporation, Redmond, Washington, DC, USA).

Results

Litter quality, environmental, and microbial parameters

All N treatment in the medium phase and intermediate-N addition increased litter residual mass ($p < 0.05$), although N treatment had no impact on the litter residual mass in the early phase ($p > 0.05$, Table 1). Litter TC, TN, TS, litter-derived DOC contents, and the ratios of C/N were similar irrespective of the treatment with the exception of significantly low C/N ratio and TC and TS concentrations under low- and intermediate-N addition in the medium phase ($p < 0.05$, Table 1). The EC and pH of the sediment were similar irrespective of the treatment except for an increase in pH values after N addition in the early phase ($p < 0.05$, Table 2). Sediment TC and TN in the medium and TC in the late phase significantly decreased under the three N treatments ($p < 0.05$, Table 2). Most of the TN and DIN of the porewater were unchanged with the exception of the intermediate-N treatment in the early phase and the high-N treatment in the late phase (Table 2). Crab hole density tended to decrease after N addition in the early phase but increased in the medium phase and was unchanged in the late phase (Table 2). In addition, except a significantly low fungal biomass in high-N treatments and a relatively low cellobiohydrolase activities in low–N treatments, the biomass of gram-positive and gram-negative bacteria, fungi, total PLFA, β-glucosidase, and cellobiohydrolase did not change significantly after N addition (Table 3).

Response of litter CO_2 evolution rates and accumulative release to N addition

During decomposition, litter CO_2 evolution rates (9.97–307.54 μg C g^{-1} h^{-1}) significantly decreased over time ($p < 0.001$, Figure 2). In most decomposition times, there was no significant difference on litter CO_2 evolution rates with N treatment or interaction term [time × treatments] (Figure 2). Litter CO_2 evolution rates showed a similar temporal pattern among different N addition treatments, in that they peaked in the early phase, weakened in the medium phase, and maintained a low level into the late phase. A notable exception was day 20, when litter CO_2 evolution rates were significantly higher in the high-N treatment relative to those of the control. Another anomaly was that higher litter CO_2 evolution rates were observed with intermediate-N levels compared with those under high-N addition at days 30 and 120 ($p < 0.05$, Figure 2).

Accumulative CO_2 release significantly increased over time ($p < 0.001$), and there was a significant difference for the interaction term [time × treatments] ($p < 0.001$) but no significant difference for N treatment over time (Figure 3A). In the early phase, there was no significant difference in accumulative CO_2 release among different N amendments (Figure 3A). By moving into the medium phase, accumulative CO_2 release significantly increased under the low- and intermediate-N amendments relative to the control; and ultimately, all N treatment significantly increased accumulative CO_2 release ($p < 0.05$, Figure 3A). At the end of our experiment, compared with the control treatment, accumulative CO_2 release increased 69.75%, 76.62%, and 39.93% for low-, intermediate-, and high-N treatments, respectively. The facilitation effect on accumulative CO_2 release by N amendments was more and more obvious over the decomposition time, especially for the low- and intermediate-N treatments (Figure 3B).

Redundancy analysis and structural equation modeling analysis

RDA results showed that litter quality and environmental and microbial parameters could explain 95.46% of the variance in CO_2 evolution rates and accumulative CO_2 release (Figure 4). Litter TC contributed the most to changes, explaining 74.6% of the variance, and was significantly positively correlated with CO_2 evolution rates. Moreover, both litter residual mass (explaining 12.8% of the variance) and sediment TN (explaining 1.6% of the variance) were positively correlated with CO_2 evolution rates, but negatively correlated with accumulative CO_2 release (Figure 4). Litter TS (explaining 1.5% of the variance) was negatively correlated with CO_2 evolution rates, however, it was positively correlated with accumulative CO_2 release (Figure 4). In addition, CO_2 evolution rates were positively correlated with porewater DIN and crab hole density (Figure 4).

The SEM of the direct and indirect effects of both sediment and porewater TN on accumulative CO_2 release showed reasonable fits ($\chi^2 = 9.33$, $p = 0.87$, GFI = 0.95, RMSEA < 0.001), and the model accounted for 87% and 71% of the variance in litter CO_2 evolution rates and accumulative CO_2 release, respectively (Figure 5). We found negative relationships between both sediment and porewater TN and sediment pH, between sediment pH and gram-negative bacteria, and between gram-negative bacteria and both litter residual mass and litter-derived DOC (Figure 5). Conversely, we observed positive relationships between sediment TN and litter residual mass, between sediment pH and litter-derived DOC, between litter residual mass and CO_2 evolution rates, and between litter-derived DOC and CO_2 evolution rates (Figure 5). In addition, both litter CO_2 evolution rates and litter production were negatively correlated to accumulative CO_2 release, whereas gram-negative bacteria and litter residual mass were positively correlated to accumulative CO_2 release (Figure 5).

TABLE 1 Litter residual mass; total carbon (TC), total nitrogen (TN), and total sulfur (TS) concentrations; dissolved organic carbon (DOC); and the ratios of TC to TN (C/N) during the decomposition phase.

Indicators	Treatments	Early phase	Medium phase	Late phase
Litter residual mass (%)	Control	44.96	20.82	10.93
	Low N	48.67	**34.20***	13.99
	Intermediate N	56.73	**40.71***	**24.76***
	High N	48.47	**35.73***	23.02
TC (mg g^{-1})	Control	382.17	305.40	202.37
	Low N	394.70	**226.07***	154.17
	Intermediate N	366.83	**218.43***	148.43
	High N	394.40	263.20	144.40
TN (mg g^{-1})	Control	15.00	16.17	13.25
	Low N	15.27	13.10	9.00
	Intermediate N	14.67	13.33	9.03
	High N	15.43	18.28	8.57
TS (mg g^{-1})	Control	1.52	2.37	2.58
	Low N	1.69	**1.60***	1.53
	Intermediate N	**1.31***	**1.66***	1.50
	High N	1.47	2.14	1.39
C/N ratio	Control	25.55	18.93	15.62
	Low N	25.88	**17.20***	16.97
	Intermediate N	24.98	**16.42***	16.34
	High N	25.61	**17.28***	16.71
DOC (mg g^{-1})	Control	7.05	2.60	1.82
	Low N	5.04	2.74	1.70
	Intermediate N	7.00	3.05	1.70
	High N	6.89	2.83	1.61

Means in bold font followed by * indicate significant differences between the given parameter and the control treatment ($p < 0.05$, ANOVA).

Discussion

Litter CO_2 evolution rates

The results of this study show that litter CO_2 evolution rates of shichito matgrass were similar to those of standing-dead leaf blades (10–295 μg C g^{-1} h^{-1}) (Kuehn et al., 2004) but higher than those of culms litter (6.38–148.01 μg C g^{-1} h^{-1}) and lower than those of leaf litter (40.37–741.91 μg C g^{-1} h^{-1}) of *Phragmites australis* (Zhang et al., 2014b). During decomposition time, litter CO_2 evolution rates peaked in the early phase, weakened in medium phase, and maintained a low-level proceeding into the late phase (Figure 2), that was consistent with Li et al. (2015). The pattern of CO_2 evolution rates could be partly explained by C availability (Figures 4, 5), the variation in litter TC and DOC concentration were in line with the trend observed for CO_2 evolution rates (Table 1). Uselman et al. (2012) suggested that approximately 36% of the DOC was either respired or stored in the early phase. This labile pool was a source of microbial respiration rates (Day et al., 2018). The downtrend in CO_2 evolution rates did not mean that microbes would die or activity decline in a short time (Li et al., 2015), and this was further confirmed by the PLFA data (Table 3). Previous studies suggested that the increasing easily-available matter (especially labile C) would accelerate the turnover of microorganisms (r-strategists, mainly bacteria) in the early phase, and later replaced by k-strategists (mainly fungi) due to growth-limiting substrate concentrations (Fontaine et al., 2003; Dilly et al., 2004; McTee et al., 2017), however, our bacteria and fungi biomass data did not show this trend during decomposition process.

Response of litter CO_2 evolution rates to nitrogen addition

Our results do not support our first hypothesis that N addition did not change litter-derived CO_2 evolution rates in most decomposition times (Figure 2). On the one hand, crab may affect litter decomposition and respiration through consumption, an increase of soil drainage and soil oxidation-reduction potential (Bertness, 1985), colonization by bacteria and resulted in a rapid decline in the C/N ratio (Werry and Lee, 2005). Since some crabs may have a preference for ingesting high N and low C/N foods, N amendments may affect the crab burrows (Nordhaus and Wolff 2007), and the increase in crab hole density and decrease in litter C/N ratio in the medium phase (Tables 1, 2) were corroborating

10.3389/fmars.2022.1035095

TABLE 2 Environmental parameters (physicochemical properties of sediment and pore water, vegetation, and crab hole density) during the decomposition phase.

Indicators	Treatments	Early phase	Medium phase	Late phase
Sediment EC (mS cm^{-1})	Control	0.30	0.41	0.55
	Low N	0.24	0.43	0.57
	Intermediate N	0.24	0.53	0.41
	High N	0.20	0.59	0.47
Sediment pH	Control	6.42	5.93	6.51
	Low N	**6.70***	6.03	5.53
	Intermediate N	**6.84***	6.21	5.53
	High N	**6.70***	6.21	5.52
Sediment TC (mg g^{-1})	Control	23.25	23.39	17.14
	Low N	20.29	**19.19***	**15.89***
	Intermediate N	19.65	**19.40***	**15.39***
	High N	19.70	**19.94***	**15.16***
Sediment TN (mg g^{-1})	Control	1.76	1.90	1.46
	Low N	1.57	**1.64***	1.50
	Intermediate N	1.59	**1.70***	1.47
	High N	1.61	**1.63***	1.45
TN of pore water (mg L^{-1})	Control	0.33	0.85	1.55
	Low N	0.28	0.77	2.33
	Intermediate N	1.00	0.26	2.12
	High N	0.31	0.77	**2.83***
DIN of pore water (mg L^{-1})	Control	0.25	0.71	0.11
	Low N	0.26	0.67	0.13
	Intermediate N	**0.95***	0.22	0.15
	High N	0.22	0.68	0.14
Crab hole density (number m^{-2})	Control	81	35	72
	Low N	75	42	55
	Intermediate N	79	**61***	34
	High N	**43***	**73***	33

Means in bold font followed by * indicate significant differences between the given parameter and the control treatment ($p < 0.05$, ANOVA).

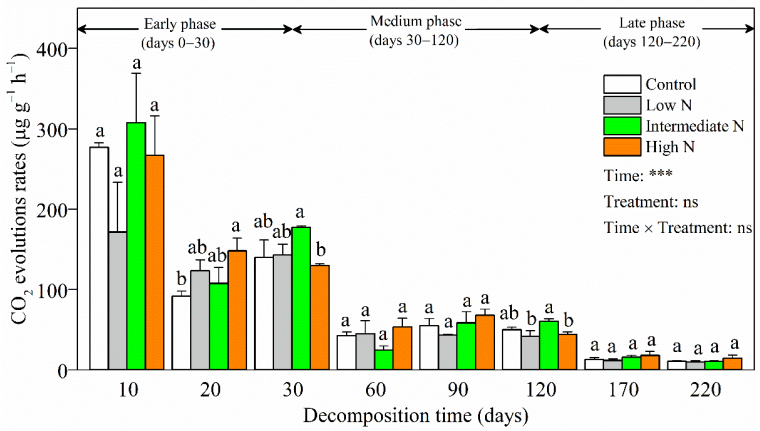

FIGURE 2
CO_2 evolution rates (mean ± SE) of C. malaccensis litter under different N addition treatments. Different letters above error bars indicate significant difference (ANOVA, $p > 0.05$). The results of repeated measure analysis of covariance are also noted and "Time × Treatment" is the combined effect of time and N treatment. *** significant at $p < 0.001$; ns, not significant.

TABLE 3 Litter phospholipid fatty acids (PLFA) and enzymes activities during the decomposition phase.

	Indicators	Treatments	Early phase	Medium phase	Late phase
PLFAs	Gram-positive bacteria biomass (nmol g^{-1})	Control	NA	89.10	99.36
		Low N	NA	95.71	112.28
		Intermediate N	36.59	100.51	86.49
		High N	40.41	104.80	94.15
	Gram-negative bacteria biomass (nmol g^{-1})	Control	NA	29.24	35.63
		Low N	NA	27.26	42.91
		Intermediate N	6.15	33.74	30.48
		High N	7.31	23.19	33.92
	Fungal biomass (nmol g^{-1})	Control	NA	8.02	8.63
		Low N	NA	8.03	10.10
		Intermediate N	2.31	8.90	9.03
		High N	2.53	**3.16***	6.54
	Total PLFA (nmol g^{-1})	Control	NA	186.35	201.99
		Low N	NA	174.18	230.65
		Intermediate N	83.55	210.73	178.81
		High N	97.22	196.15	201.76
Enzymes	β-glucosidase (nmol h^{-1} g^{-1})	Control	347.00	9.61	5.53
		Low N	288.42	8.65	10.53
		Intermediate N	366.80	12.50	4.39
		High N	351.79	15.59	14.02
	Cellobiohydrolase (nmol h^{-1} g^{-1})	Control	103.92	1.51	6.29
		Low N	**71.03***	0.65	12.21
		Intermediate N	129.04	0.69	7.20
		High N	104.10	0.71	11.63

NA, no data available.
Means in bold font followed by * indicate significant differences between the given parameter and the control treatment ($p < 0.05$, ANOVA).

these results. On the other hand, available C sources could facilitate microbial respiration initiated by high β-glucosidase activity (Badiane et al., 2001; Sinsabaugh et al., 2009; Turner and Wright, 2014). The studied N amendments had little effect on β-glucosidase, further led to a similar litter DOC for microbial respiration (Tables 1 and 3), although low litter TC and sediment TC were found after N addition in the medium phase. Eventually, although low litter TC and sediment TC may decrease litter-derived CO_2 evolution rates after N addition in the medium phase, these negative effects may offset by the positive effect of crab activity.

Interestingly, litter CO_2 evolution rates were significantly higher in the high-N treatment relative to the control in day 20 (Figure 2), this unexpected result indicated that high-N may be explained by sediment pH (Table 2 and Figure 5). In the early phase, litter leaching would increase in pH (Nykvist, 1963), consistent with our findings. In weak acidic or neutral environments (pH 6.19–7.13), acidobacteria subgroups 4 and 6 had higher sediment pH values (Jones et al., 2009; Keyport et al., 2019). These increases in relative pH may indirectly increase microbial respiration (Tables 2, 3 Figure 5).

By moving into the late phase, traditionally, litter remains were relatively refractory materials (i.e., lignin), and lignin-degradation rates regulated litter decomposition (Valiela et al., 1985; Berg and Matzner, 1997). The refractory lignin was decomposed by white-rot basidiomycetes and phenol oxidase, and they were greatly decreased by increased N availability (Dix and Webster, 1995; Carreiro et al., 2000; Gallo et al., 2005). Previous studies have suggested that N has a retarding effect on decomposition in the late phase when significant negative correlations were noted between N content in humus and respiration rate (Berg and Matzner, 1997). Our results challenge the traditional view that there was no significant difference in litter CO_2 evolution rates among the different N addition treatments in the late phase.

Response of litter accumulative CO_2 release to nitrogen addition

Our data supports hypothesis 2 based on the significant increase in accumulative CO_2 release in the medium and late phase, though this motivating effect was not observed in the early phases (Figure 3B). Rubino et al. (2010) reported the fraction of litter C released as CO_2 to the atmosphere to be nearly 30% of the total litter C loss. Based on this estimate, litter

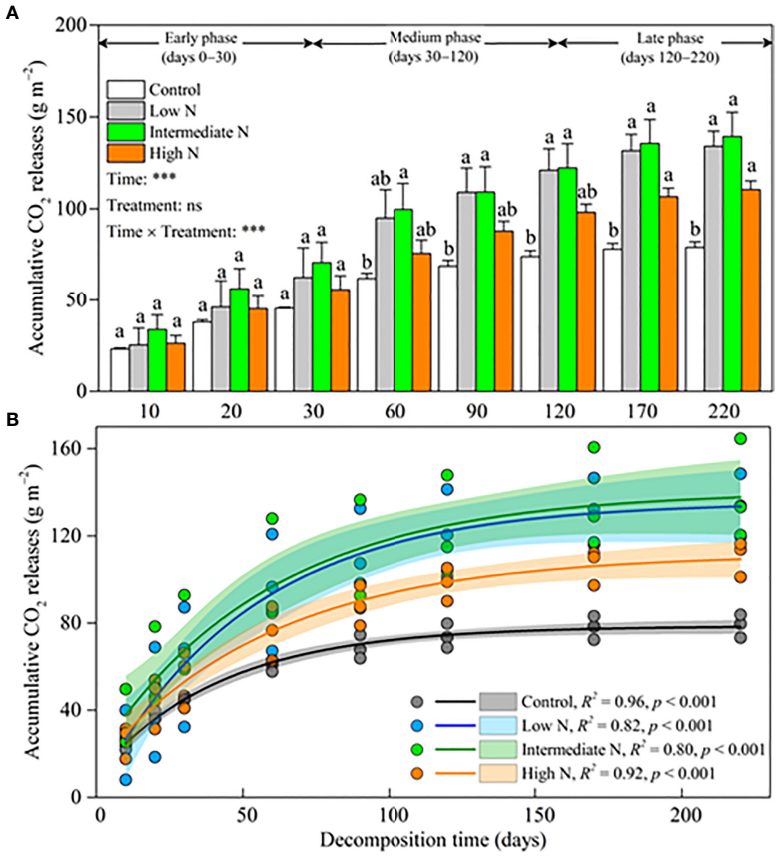

FIGURE 3
(A) Litter accumulative CO_2 release (mean ± SE) under different N addition treatments. Different letters above error bars indicate significant difference (ANOVA, $p > 0.05$). The results of repeated measure analysis of covariance are also noted, and "Time × Treatment" is the combined effect of time and N treatment. ** significant at $p < 0.01$; *** significant at $p < 0.001$; ns, not significant. **(B)** exponential regressions under different N addition treatments. The scatters are litter accumulative CO_2 release (three duplicates). The lines are asymptotic for exponential regressions. Shaded areas are 95% confidence intervals. R^2 and p values are from exponential regressions.

CO_2 release could reach 53% of the total litter C loss under intermediate-N deposition (16 g N m^{-2} yr^{-1}). The accumulative CO_2 released from litter was directly affected by litter CO_2 evolution rates, litter residual mass, and litter production (Figure 5). Because of no significant litter CO_2 evolution rates under different N treatments, we suggested that litter production and residual mass were the most important factors regulating accumulative CO_2 release.

Meta-analysis results indicated that N addition significantly increased aboveground biomass and litter input by 31% and 20%, respectively (Liu and Greaver, 2010; You et al., 2017). A similar relationship was found in our studies. Not only N fertilization increases litter production, but also ameliorates litter chemistry (Wedin and Tilman, 1996; Gerdol et al., 2007). Higher N availability increased plant lignin and protein, but decreased plant hemicellulose (Liu et al., 2016). On the other hand, litter residual mass increased after N addition in the medium and late phases. The retarding effect of increasing N on decomposition in the late phase has been demonstrated by previous studies, and there are four possible nonexclusive explanations: (i) decreasing sediment pH, further decreasing bacterial diversity (Geisseler and Scow, 2014); (ii) directly inhibiting the decay of lignin and cellulose (Tu et al., 2011); (iii) decreasing white-rot basidiomycetes and phenol oxidase, resulting in slow lignin decomposition (Dix and Webster, 1995; Carreiro et al., 2000; Gallo et al., 2005); (iv) causing more humus to be left, and the resulting lower levels of Mn further retards humus decomposition (Berg and Matzner, 1997). Ultimately, the combined effect of the increased litter input and the inhibiting effect of external N on litter decomposition increased accumulative CO_2 release (Figure 6).

Uncertainties and future study

To the best of our knowledge, our study was the first time to investigate the response of litter CO_2 evolution rates and

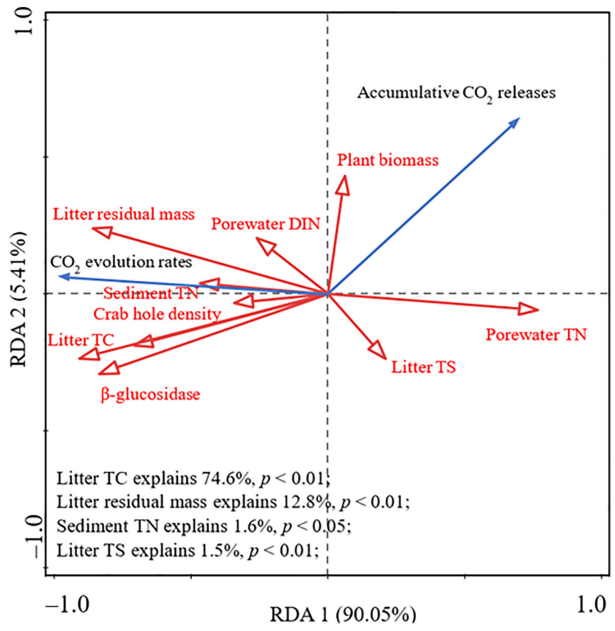

FIGURE 4
Redundancy analysis (RDA) ordination plots for the first two principal dimensions of the relationship among CO_2 evolution rate, accumulative CO_2 release, environmental parameters, microbial biomass, and litter matrix.

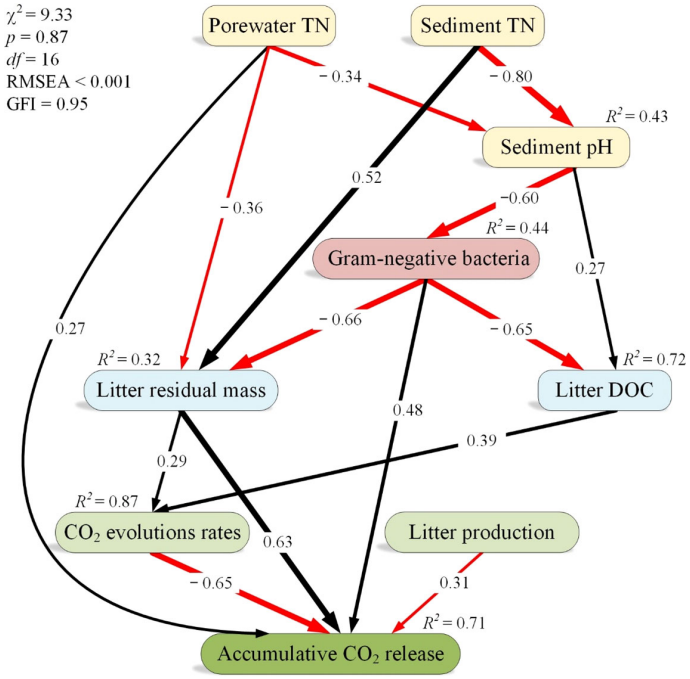

FIGURE 5
A structural equation model analysis of the effects of porewater total nitrogen (TN) on accumulative CO_2 release. The width of arrows indicates the strength of the standardized path coefficient. Black lines indicate positive path coefficients, while red lines indicate negative path coefficients ($p < 0.05$). R_2 values associated with response variables indicate the proportion of variation explained by relationships with other variables. GFI, goodness-of-fit index; RMSEA, root mean square errors of approximation; χ^2, chi-square tests.

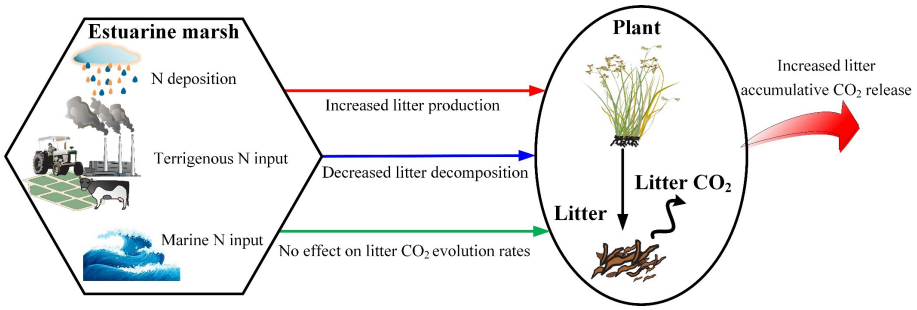

FIGURE 6
Conceptual schematic of the effect of N load on litter accumulative CO_2 release in estuarine marshes.

accumulative release to N addition in a subtropical estuarine marsh. Nevertheless, there remains a great deal of uncertainty in our results. Our data highlights the role of litter production and decomposition as important environmental factors influencing litter CO_2 production and emissions. The increase in litter input and inhibition of litter decomposition by N loading increased accumulative CO_2 release. This indicates that N input could greatly cause loss of litter C as CO_2 at the regional level and globally, particularly in tidal freshwater marshes. Previous studies have demonstrated that increases in sediment CO_2 emissions induced by N loads were counteracted by sea-level rise and the subsequent inhibition of the increase in N on C emissions (Hu et al., 2019a); to some extent, the litter CO_2 emissions played a similar role. Our findings provide a comprehensive perspective for understanding the underlying response of litter CO_2 release to N addition in an estuarine marsh, and thus, improve predictions and climate adaptation strategies.

Owing to the limitations of the study region, we selected litter of shichito matgrass for analysis. Nitrogen deposition is an ongoing process. Since litter quality, especially plants N matrix (i.e., C/N), is crucial for litter CO_2 evolution and decomposition (Chambers et al., 2001; Uselman et al., 2012; Zhang et al., 2014b; Day et al., 2018; Jacobs et al., 2018), the CO_2 evolution and release from litter of different vegetation types and the initial litter produced under different N deposition conditions are needed to investigate in further studies. In addition, the N added in the sample plots may be difficult to infiltrate or easily carried away by the tides. Therefore, it is necessary to carry out a microcosms experiment.

Conclusions

Our results, overall, provide a basis for developing guidelines for CO_2 emission predictions. Litter CO_2 evolution rates peaked in the early phase, weakened in the medium phase, and maintained a low-level proceeding into the late phase. Litter-derived CO_2 evolution rates were similar after N addition in most decomposition times. Although low litter TC and sediment TC may decrease litter-derived CO_2 evolution rates after N addition in the medium phase, these negative effects may offset by the positive effect of crab activity. Generally, the average accumulative CO_2 release increased after N addition, mainly driven by litter production and decomposition. Our results indicated that an increase in N load significantly increased the litter CO_2 release, and thus, improves predictions and provides key information for developing climate adaptation strategies.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

WH: Writing – Original Draft, Conceptualization, Software, Formal analysis, Data Curation. CZ, CT, and GL: Conceptualization, Methodology, Writing – Review & Editing. XL, JZ, and MZ: Methodology, Software, and Formal analysis. YC and LZ: Writing – Review & Editing, Project administration, Funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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