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Influences of chloroform exposure time and soil water content on C and N release in forest soils

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Abstract

We investigated the influence of fumigation conditions of the chloroform fumigation–extraction method on the release of extractable carbon (C) and nitrogen (N) from organic and mineral soil horizons of 11 mature forests. Soil samples were fumigated with chloroform vapor for 1, 3, 5, or 10 d at two different water contents: field moist or field capacity (−33 kPa matric potential). We found that for approximately half our soils, 0.5 M K_2SO_4-extractable N and C reached a maximum after 1 d of fumigation. The effect of soil wetting on C and N flushes from O horizons was variable but in mineral soils, increasing the soil water content generally resulted in more extractable C and N following fumigation. For the majority of soils assessed, increasing the soil water content did not change the fumigation time necessary to generate the maximum C or N flush. Observed changes in the C/N ratios of the fumigation flush with changes in fumigation time and the sensitivity of this C/N ratio to changes in soil water content suggest that different fumigation conditions may result in different organic pools being extracted. These effects appear to be extremely soil specific. We recommend that the effects of fumigation time and water content be evaluated before the C and N flushes from the fumigation extraction method are used to assess differences in microbial C and N among contrasting forest soils.

Keywords: Carbon; Chloroform-fumigation; Microbial biomass; Nitrogen

1. Introduction

The microbial biomass is a vital soil component, acting as a source and a sink for plant available nutrients, as well as catalyzing the transformations of these nutrients in soil. Because of the importance of this soil pool, a variety of methods have been developed to estimate the size and, in some cases, the chemical composition of the soil microbial biomass. These methods include direct microscopy, as well as a host of chemical extraction, biological incubation, and physiological approaches (Paul and Clark, 1996).

Over the past 25 years, numerous studies have used chloroform ($CHCl_3$)-based methods to estimate the carbon (C) and nitrogen (N) contents of the microbial biomass in soil. These methods have been the preferred choice of many investigators because of their ease of use, and the ability to assess the quantities of elements contained within the microbial biomass. Chloroform-based methods take two fundamental forms: the fumigation incubation (FI) method and the fumigation extraction (FE) method. In the FI method, soils are typically exposed to $CHCl_3$ vapor for 1 d, and then the fumigant is removed and the soils incubated for 10 d along with or without unfumigated control soils (Horwath and Paul, 1994). The net release of CO_2–C and NH_4^+–N, based either on the fumigated soil alone or relative to the incubated control soil, during the 10 d incubation period is used to estimate the size of the microbial biomass C and N pools, respectively. In the FE method, soils are also generally exposed to $CHCl_3$ for 1 d and the fumigant subsequently removed, but then the soils are immediately extracted with a K_2SO_4 solution (Horwath and Paul, 1994). The net organic C and total N released due to $CHCl_3$ exposure is used to estimate the size of the microbial biomass C and N pools, respectively. The FE method has become the favored technique of the two $CHCl_3$ approaches because of its shorter analysis time, and its applicability to

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soils with low pH or high organic matter contents, saturated and dry soils without a 'pre-incubation,' and soils that have recently received fresh substrates (Brookes et al., 1985b; Vance et al., 1987; Jenkinson, 1988; Tate et al., 1988; Sparling and West, 1989; Ross, 1990).

In spite of the many advantages of the FE method, several problems with the technique exist. For example, the 1 d fumigation recommended by Brookes et al. (1985b) for estimating microbial N was based in part on their observation that maximum release of microbial N is reached after a 5 d fumigation, and that a consistent relationship exists between N flushes after 1 and 5 d. However, some investigators have found that these conditions are not frequently met (Davidson et al., 1989; Ross and Tate, 1993). Additionally, like the FI method, the FE method is sensitive to changes in soil water content (Sparling and West, 1989; Sparling et al., 1990). Nevertheless, some studies suggest that this sensitivity may only occur in very dry soils, which would preclude the need under most conditions to make these time-consuming water adjustments (Sparling and West, 1989). Finally, Horwath and Paul (1994) suggested that the FI and FE methods may assess different soil organic C pools based on the specific ^{14}C activities of the flushes released from soils labeled with ^{14}C plant residues. It is also possible that different exposure times of a soil to $CHCl_3$ results in the extraction of different soil organic matter pools by the FE method. If this is the case, the C/N ratio of the fumigation flush may also change.

The objectives of our studies of the FE method for assessing microbial C and N were: (1) to determine the shortest fumigation time necessary for achieving the largest and most consistent C and N release; (2) to assess how changes in soil water contents found under field conditions affect the C and N release by fumigation; and (3) to test the fidelity of the FE method for measuring a single, well-mixed pool of C and N in the soil by evaluating changes in the C/N ratio of the FE flush resulting from different fumigation time. We addressed these objectives using organic and mineral soil samples from a wide range of forest types.

2. Materials and methods

2.1. Soil sampling

Organic layers (O horizons) and mineral soil from A horizons were collected from 11 mature forest sites in Oregon and New Mexico, USA, which span the range of net primary production in North American forests (Stark and Hart, 1997; Table 1). Samples were collected in May and June 1993, except for two sites (the New Mexico Spruce/Fir and Oregon mountain hemlock sites) that were sampled in August and September 1993 because they were beneath snow through June. Five sampling points were located at random within each site, and from these points we collected the organic horizon from approximately a 25×25 cm^2 area. At the New Mexico Pinyon–Juniper site, separate samples were collected from beneath Pinyon and Juniper canopies. Organic horizon densities varied from 0.48 to 9.6 kg dry mass/m^2 across the sites. Mineral soil samples were collected from 0 to 15 cm depth by pounding 5 cm diameter, thin-walled polycarbonate pipe into the soil. Samples were placed in plastic bags, transported to a laboratory at Northern Arizona University in a cooler containing ice and stored at 4 °C for less than 6 months until analyses were performed.

Immediately before initiating the study, replicate soil samples were composited by horizon and site. Organic horizon materials were cut into pieces <2 cm in length with scissors and mixed by hand. All material >1 cm diameter was discarded. Mineral soils were sieved at field moisture through a 4 mm sieve.

2.2. Experimental design and analytical procedures

Eight treatment combinations were applied to each of the 23 organic and mineral soil materials. The treatment combinations consisted of two soil water contents crossed with four fumigation times. Three replicates were used for all soil type and treatment combinations.

After homogenization of the samples, subsamples were taken from each soil composite ($\sim$13 g dry weight for mineral soil, and $\sim$4.0 g for O horizon material). One set of subsamples was left at their original field water content and one set was adjusted to field capacity (-33 kPa matric potential). Samples were adjusted to field capacity by placing them in Büchner funnels with a pre-wetted, Whatman No. 42 filter paper at the bottom, and then adding a saturating amount of deionized water to the sample while applying a 33 kPa vacuum (Topp et al., 1993). Field capacity was assumed to have reached when the soil no longer eluted water in response to the vacuum.

Immediately following adjustment of the water contents, samples were weighed in 50 ml glass beakers, which were placed in glass vacuum desiccators for exposure to $CHCl_3$. Approximately 50 ml of ethanol-free, hydrocarbon-stabilized $CHCl_3$ were poured into beakers containing boiling chips and one beaker was placed into each desiccator. Paper towels moistened with deionized water were also placed in each desiccator to help maintain the water content of the soils during fumigation. Four desiccators were assigned to each of the four fumigation time treatments (1, 3, 5, and 10 d). One set of soil samples was immediately extracted to serve as an unfumigated control (0 d exposure). The desiccators were evacuated until the $CHCl_3$ boiled. Room air was permitted back into the desiccator, and the process was repeated three more times to promote distribution of the $CHCl_3$ vapor into micropores of the soil samples. The desiccators were left evacuated the last time.

Table 1
Selected characteristics of study sites in New Mexico and Oregon

Ecosystem	Site key	Soil texture (kg/kg)		Gravimetric water content (kg/kg)				pH		Total C (g/kg)		Total N (g/kg)		CEC (cmol/kg)	
		Silt	Clay	O		A		O	A	O	A	O	A	O	A
				M	C	M	C								
New Mexico sites															
Pinyon/Juniper (*Pinus edulis/Juniperus osteosperma*)	PJ	20	16	0.05 (p) 0.03 (j)	0.57 0.74	0.04	0.19	5.0 (p) 7.5 (j)	6.1	183	19.2	6.7	1.1	46.6 (p) 51.2 (j)	16.6
Ponderosa pine (*Pinus ponderosa*)	NP	37	11	0.18	0.85	0.06	0.15	5.3	5.0	357	14.8	13.5	0.5	63.6	6.5
Mixed conifer (*Pseudotsuga menziesii/Abies concolor*)	MC	32	16	0.42	0.77	0.13	0.21	5.8	5.1	208	26.5	8.4	1.1	55.9	19.2
Aspen (*Populus tremuloides*)	A	38	15	0.82	0.93	0.21	0.27	5.4	5.1	301	24.3	16.5	1.4	60.7	17.7
Spruce/Fir (*Picea engelmanii/Abies lasiocarpa*)	SF	31	19	0.67	0.97	0.18	0.26	5.2	4.6	254	43.6	11.6	2.0	57.2	12.9
Oregon sites															
Western Juniper (*Juniperus occidentalis*)	J	20	14	0.67	0.91	0.09	0.17	7.2	5.9	360	5.6	14.3	0.5	76.2	11.1
Ponderosa pine (*Pinus ponderosa*)	OP	19	9	0.50	0.86	0.12	0.18	4.6	5.5	344	39.4	7.7	0.8	42.1	9.3
Mountain hemlock (*Tsuga mertensiana*)	MH	25	16	0.82	1.05	0.31	0.54	4.4	4.8	316	72.4	11.2	2.1	39.7	12.1
Douglas-fir (*Pseudotsuga menziesii*)	DF	52	26	0.65	0.79	0.13	0.24	5.9	5.0	236	24.3	8.9	1.7	61.5	14.5
Red alder/Douglas-fir (*Alnus rubra/Pseudotsuga menziesii*)	RD	46	26	1.15	1.33	0.68	0.70	3.8	3.5	437	170	22.1	6.8	40.1	32.5
Western hemlock/Sitka spruce (*Tsuga heterophylla/Picea stichensis*)	HS	49	37	1.08	1.19	0.71	0.77	3.5	3.8	400	134	12.5	5.7	45.0	32.5

O = O horizon; A = A horizon (top 15 cm); M = field moisture; C = field capacity; j = O horizon under Juniper trees; p = O horizon under Pinyon trees; pH measured in 0.01 M $CaCl_2$.

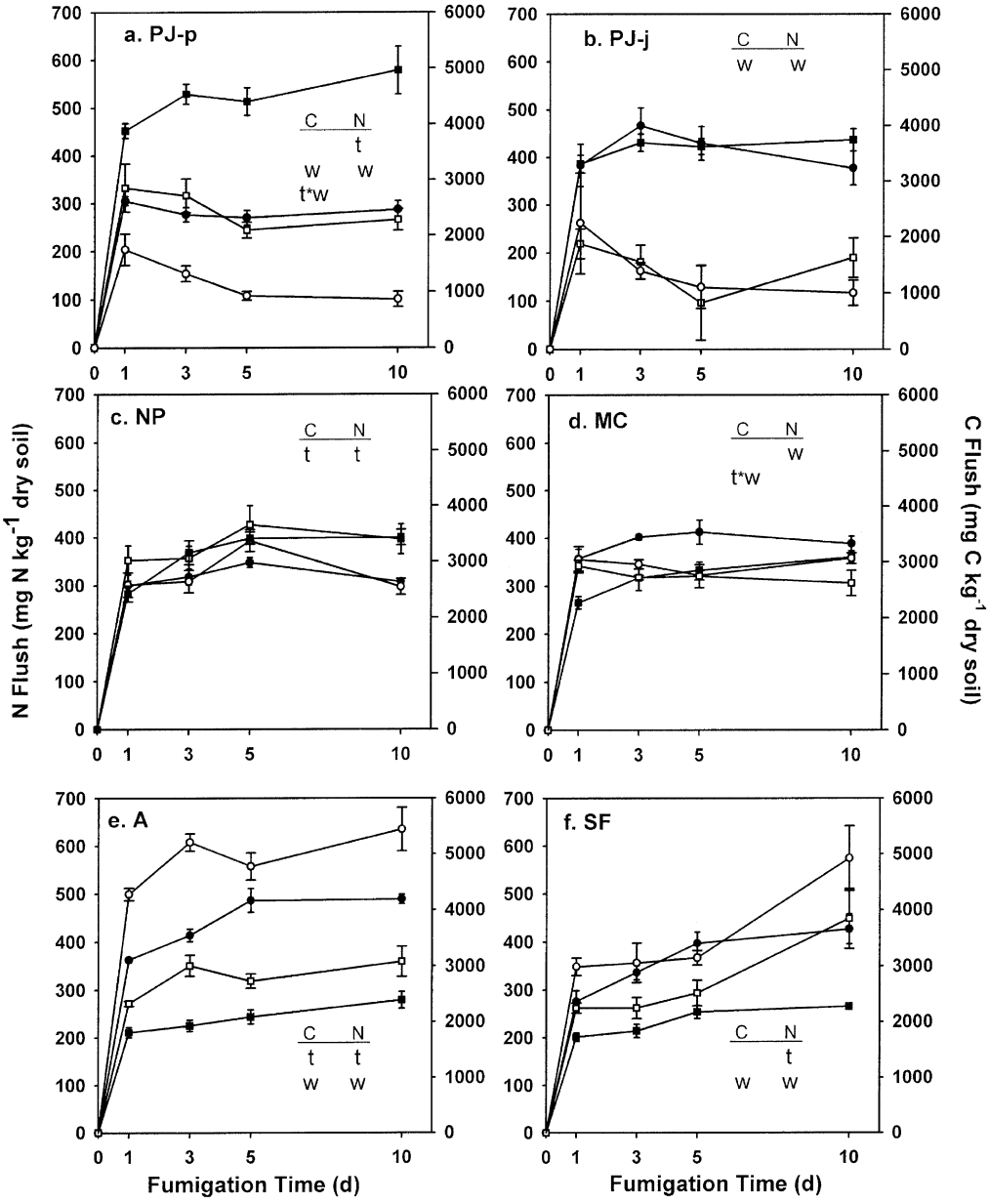

Fig. 1. Extractable C and N flushes from O horizons taken from New Mexico sites after 1, 3, 5, and 10 d of exposure to chloroform. Each data point (■ = field capacity, C flush; □ = field-moist, C flush; ● = field capacity, N flush; ○ = field-moist, N flush) represents the mean of three replicates ± one SEM. Letters in figure indicate whether time (t), water (w), or time × water ($t \times w$) were significant ($p < 0.05$) for C and N. Order of panels follows elevational gradient from lowest (Pinyon–Juniper) to highest site (Spruce–Fir). See Table 1 for key to site labels.

After fumigation, all samples (including unfumigated controls) were extracted in 100 ml of 0.5 M K_2SO_4, shaken for 1 h on a mechanical shaker, then filtered through Whatman No. 1 filter paper (pre-leached with deionized water). Total organic C concentrations in extracts were determined by ultraviolet-enhanced persulfate oxidation using a Dohrmann DC-80 Carbon Analyzer with an infrared detector (Tekmar-Dohrmann, Cincinnati, OH, USA). Total N concentrations in the extracts were determined by micro-Kjeldahl digestion, modified to exclude NO_3^- (Pace et al., 1982; Davidson et al., 1989). Ammonium-N in digested samples was then analyzed colorimetrically using the salicylate method (Lachat instruments, Inc., 1992) and a Lachat AE Flow-injection Autoanalyzer (Lachat Instruments, Milwaukee, WI, USA). The C flush due to fumigation was calculated by subtracting the concentration of total organic C in extracts of the unfumigated subsample from the concentration in extracts of the respective fumigated subsample. The N flush was calculated in the same manner but using the total N concentration contained in the extracts. Gravimetric soil water content was determined for each composite sample (70 °C for organic

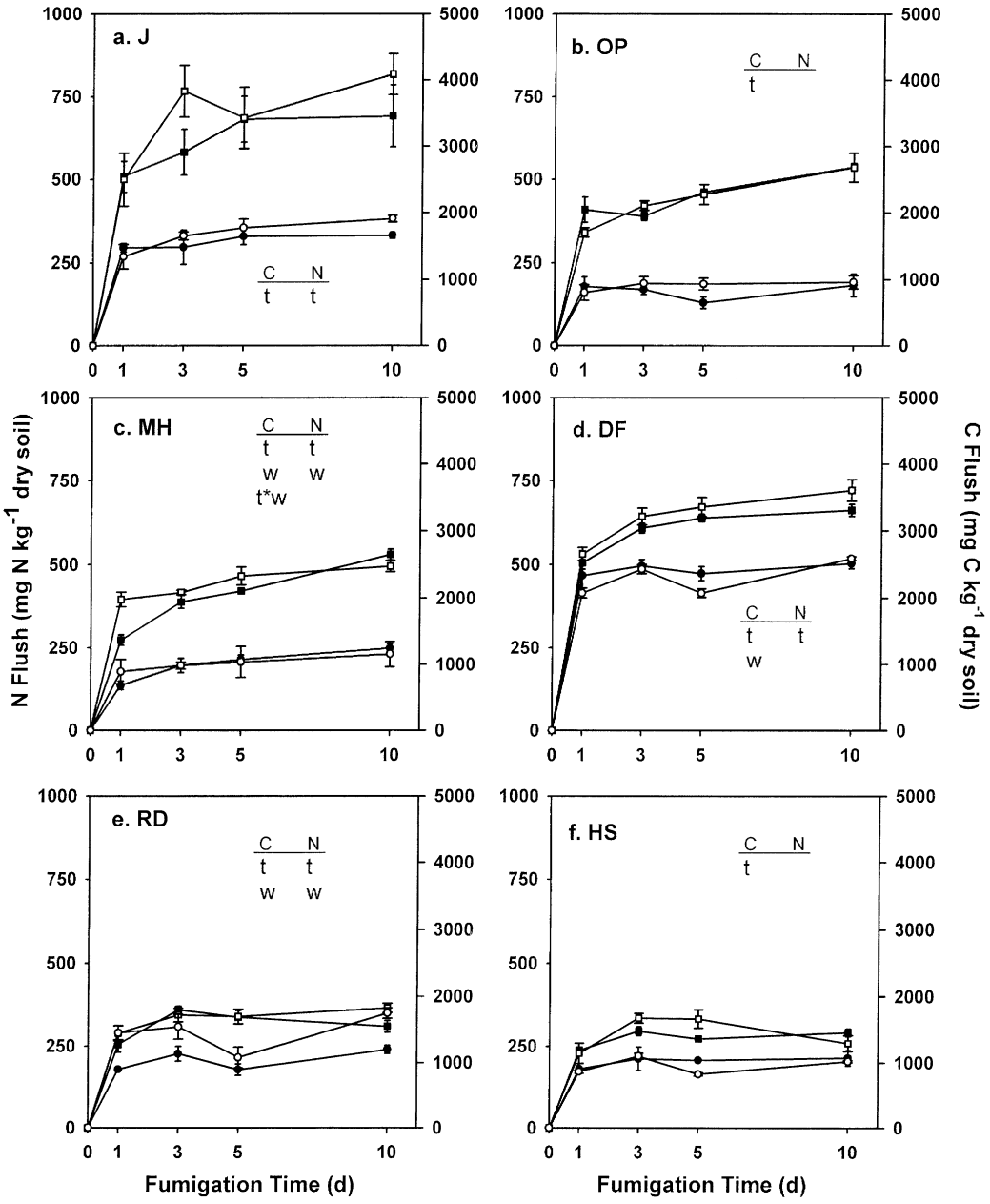

Fig. 2. Extractable C and N flushes in O horizons taken from Oregon sites after 1, 3, 5, and 10 d of exposure to chloroform. Each data point (■ = field capacity, C flush; □ = field-moist, C flush; ● = field capacity, N flush; ○ = field-moist, N flush) represents the mean of three replicates ± one SEM. Letters in figure indicate whether time (t), water (w), or time × water ($t \times w$) were significant ($p < 0.05$) for C and N. Order of panels follows longitudinal gradient from most interior site (Juniper) to coastal site (Western hemlock/Sitka spruce). See Table 1 for key to site labels.

horizon material and 105 °C for mineral soil). All C and N flush data are expressed as mg C or N kg^{-1} oven-dry soil.

2.3. Statistical analyses

Data on C, N, and the C/N ratio of fumigation flushes were subjected to analyses of variance (ANOVA), with fumigation time and soil water content as fixed main effects. Separate ANOVAs were performed for soils from each horizon within each site. Bonferoni-adjusted pairwise comparisons were used to assess when maximum C and N flushes occurred as a function of fumigation time and soil water content.

We assumed that a 1 d exposure to $CHCl_3$ was adequate for producing the maximum C and N flush for a given soil type when: (1) there was no significant effect of time ($p > 0.05$); or (2) there was either a significant time effect or a significant time × soil water content interaction, and post-hoc tests showed that the flush after 1 d was the highest or not significantly different from the highest. We used analysis of covariance (ANCOVA) to test the effect of soil water content on the relationship between 1 and 5 d flush

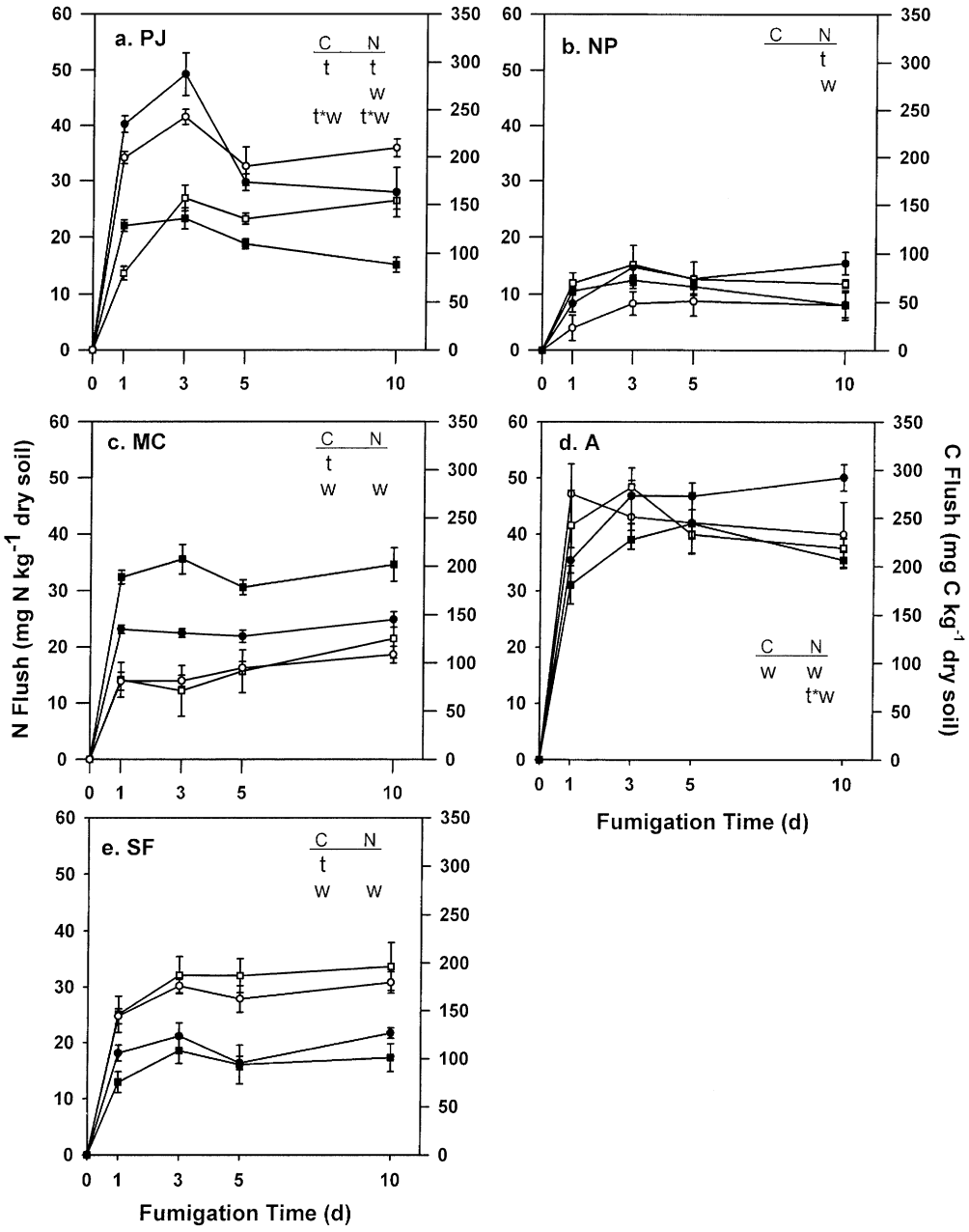

Fig. 3. Extractable C and N flushes in top 15 cm of mineral soil (A horizon) taken from New Mexico sites after 1, 3, 5, and 10 d of exposure to chloroform. Each data point (■ = field capacity, C flush; □ = field-moist, C flush; ● = field capacity, N flush; ○ = field-moist, N flush) represents the mean of three replicates ± one SEM. Letters in figure indicate whether time (t), water (w), or time × water ($t \times w$) were significant ($p < 0.05$) for C and N. Order of panels follows elevational gradient from lowest (Pinyon–Juniper) to highest site (Spruce–Fir). See Table 1 for key to site labels.

values. All statistical analyses were conducted using SYSTAT V (SPSS, 1997).

3. Results

3.1. Effects of fumigation time and soil water content on C and N flushes

In general, O horizon samples required longer fumigation time for maximum release of C compared to A horizon samples. For most sites, this effect was irrespective of soil water content (except at site MC, where there was a significant time × soil water content interaction term [$p < 0.05$]). For O horizons, a 1 d fumigation was sufficient for maximum release of C from only three of the 12 samples (sites PJ-p, PJ-j, MC [field-moist] and SF), whereas six sites required as much as a 10 d fumigation (sites MC [field-capacity], A, J, OP, MH, and DF). The three remaining samples required 3 d (sites RD and SH) or 5 d fumigation times (site NP) (Figs. 1 and 2). In the A horizon, however, a 1 d fumigation released the maximum

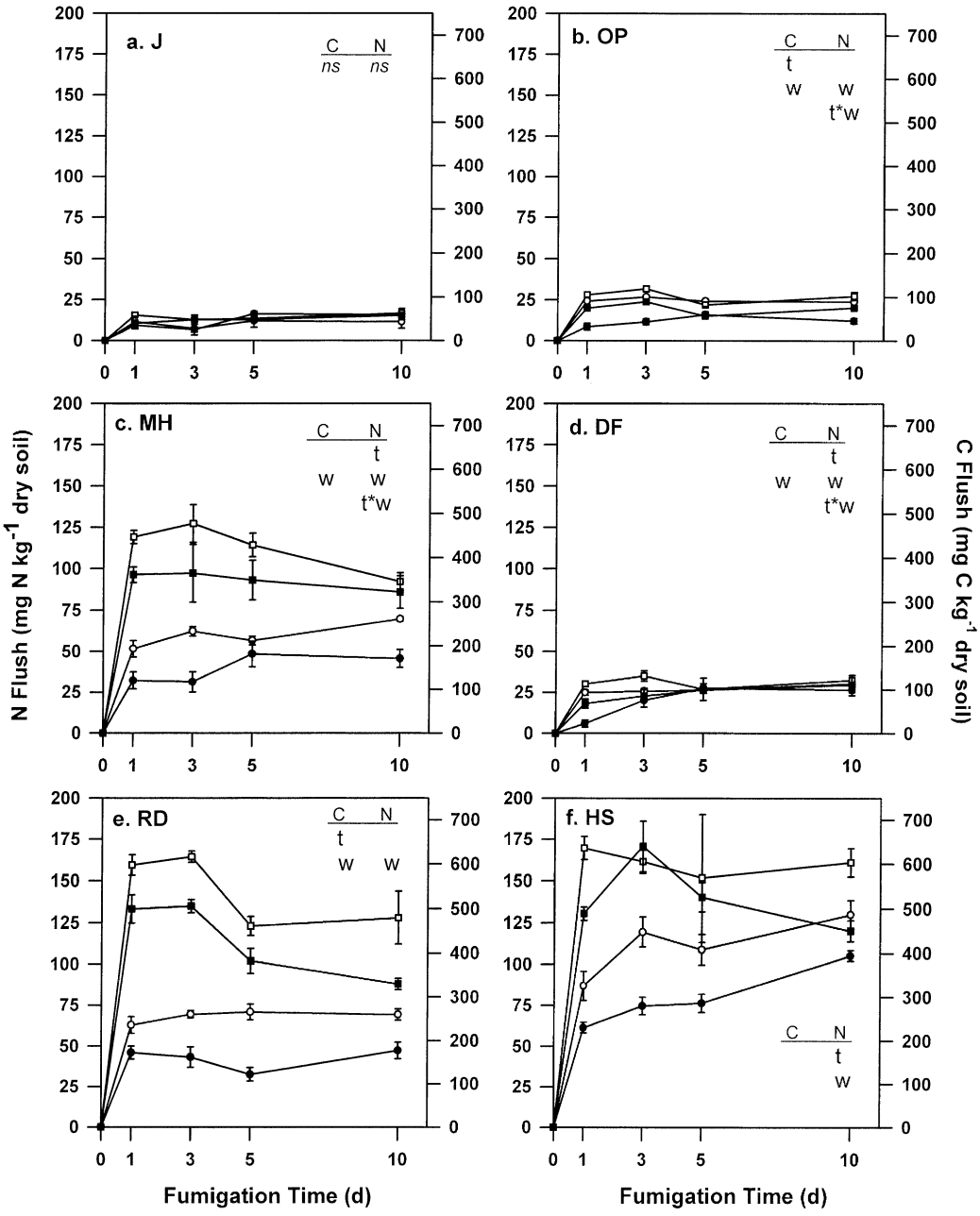

Fig. 4. Extractable C and N flushes in top 15 cm of mineral soil (A horizon) taken from Oregon sites after 1, 3, 5, and 10 d of exposure to chloroform. Each data point (■ = field capacity, C flush; □ = field-moist, C flush; ● = field capacity, N flush; ○ = field-moist, N flush) represents the mean of three replicates ± one SEM. Letters in figure indicate whether time (t), water (w), or time × water ($t \times w$) were significant ($p < 0.05$) for C and N. Order of panels follows longitudinal gradient from most interior site (Juniper) to coastal site (Western hemlock/Sitka spruce). See Table 1 for key to site labels.

amount of C from nine of the 11 sites in the A horizon (Figs. 3 and 4). For the other two sites (MC and SF, on the New Mexico transect), a 10 d fumigation was necessary for maximum release of C.

Release of soil N in mineral soils generally followed a pattern similar to that of C release, where 1 d fumigation was adequate to achieve the maximum N flush for most samples. As with C release in the O horizon, N release from O horizons typically required longer fumigation periods. A 1 d exposure to $CHCl_3$ was adequate to release the maximum amount of N for five of the 12 O horizons (sites PJ-p, PJ-j, MC, OP, and HS; Figs. 1 and 2), and nine of the 11 A horizons (sites PJ, MC, A, SF, J, OP, MH, DF, RD; Figs. 3 and 4).

In most cases, soil water content had a significant effect on C and N flushes, but the direction of the influence appeared to depend, in part, on soil horizon. Carbon flushes from O horizon materials showed a variable response to increases in soil water content: two sites had higher flushes (PJ-j, RD), three sites had lower flushes (A, SF, and DF) and

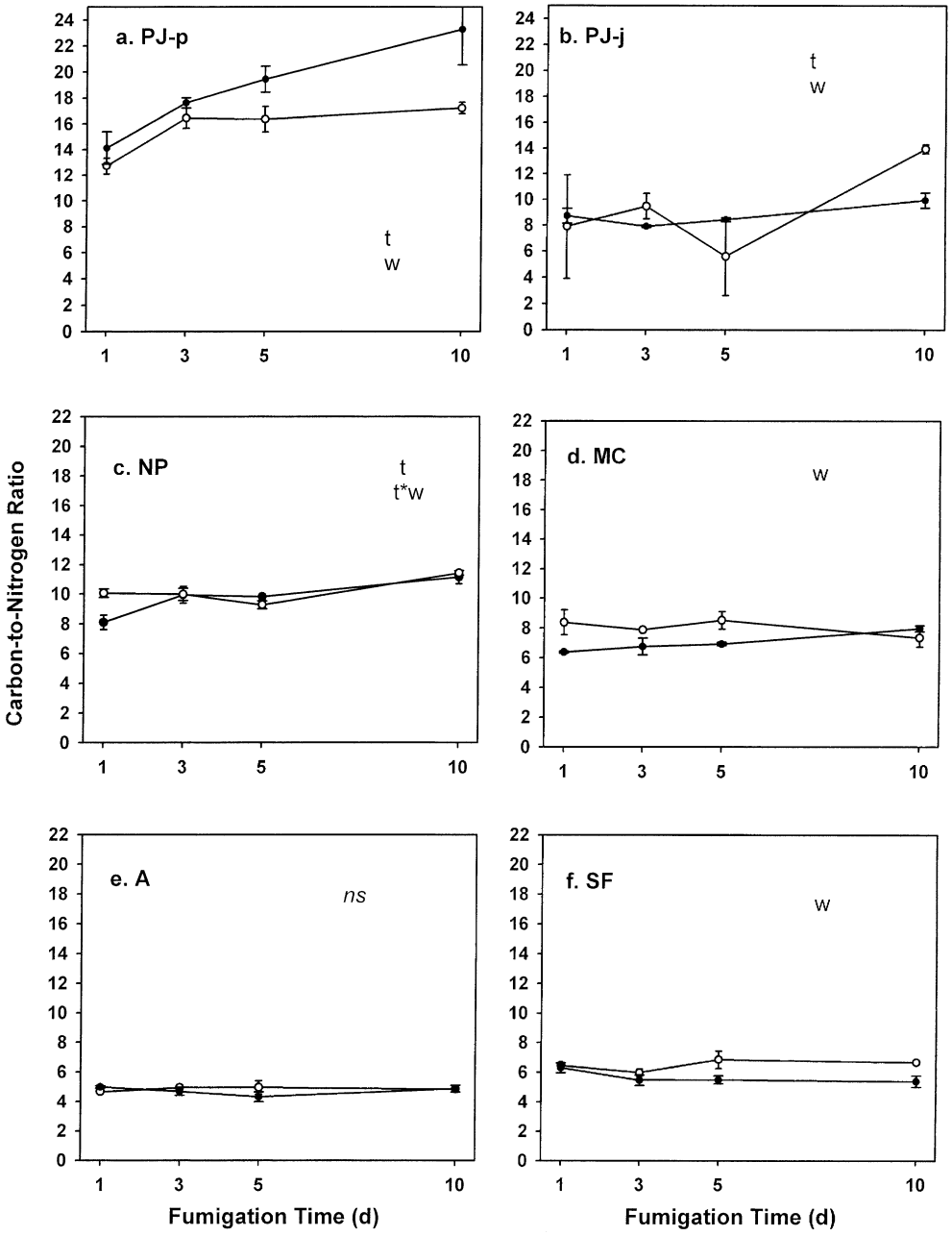

Fig. 5. Extractable carbon-to-nitrogen (C/N) flush ratios in O horizons taken from New Mexico sites after 1, 3, 5, and 10 d of exposure to chloroform. Each data point (● = field capacity; ○ = field-moist) represents the mean of three replicates ± one SEM. Letters in figure indicate whether time (t), water (w), or time × water ($t \times w$) were significant ($p < 0.05$); ns = not significant. Order of panels follows elevational gradient from lowest (Pinyon–Juniper) to highest site (Spruce–Fir). See Table 1 for key to site labels.

four sites showed no response (NP, J, OP, and HS) (Figs. 1 and 2). The remaining sites (PJ-p, MH and RD) had significant time × soil water content interaction terms ($p < 0.05$). The sites followed the same pattern for N flushes from O horizons, with two exceptions. At site DF, increased water content resulted in decreased C flush, but had no effect on N flush (Fig. 2d). At the RD site, water content increased C flush but decreased N flush (Fig. 2e).

In A horizon samples, there was less variability than in O horizon samples. Most sites (where increased water content had a significant effect) showed increased C and N flushes with increased soil water contents (Figs. 3 and 4).

The effect of soil water content interacted with the effect of $CHCl_3$ exposure time at a number of sites. This 'time × water' interaction was observed mainly for N flushes in soils taken from A horizons (sites PJ, A, OP, MH, and DF; Figs. 3 and 4). In four of the five cases (except for MH site), N flush in wetter soils peaked later than their drier counterparts. No O horizon material had a significant time × water interaction for N flush (Figs. 1 and 2). For C

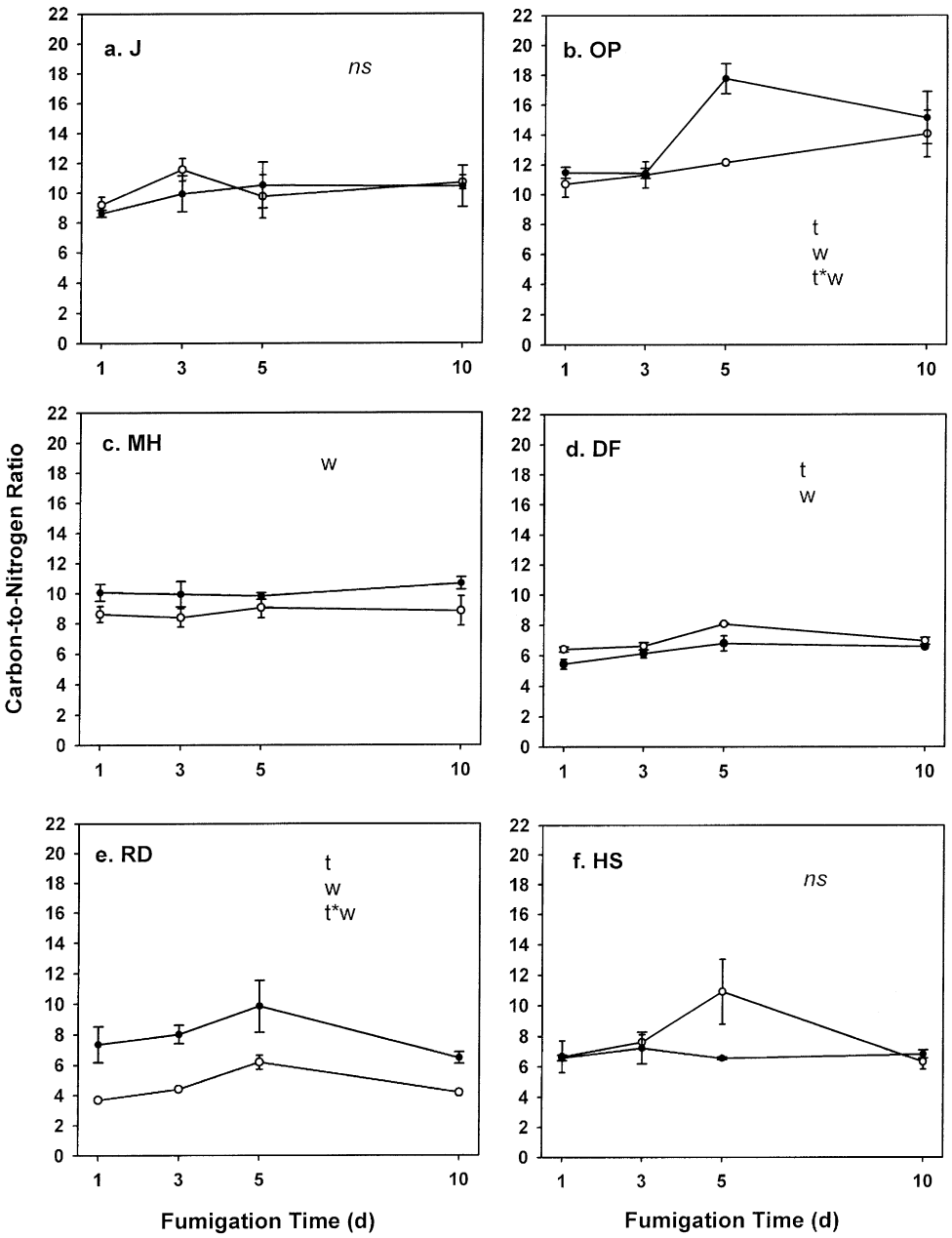

Fig. 6. Extractable carbon-to-nitrogen (C/N) ratios in O horizons taken from Oregon sites after 1, 3, 5, and 10 d of exposure to chloroform. Each data point (● = field capacity; ○ = field-moist) represents the mean of three replicates ± one SEM. Letters in figure indicate whether time (t), water (w), or time × water ($t \times w$) were significant ($p < 0.05$); ns = not significant. Order of panels follows longitudinal gradient from most interior site (Juniper) to coastal site (Western hemlock/Sitka spruce). See Table 1 for key to site labels.

flushes, two samples of O horizon materials (sites PJ-p and MH, Figs. 1 and 2) and one sample of A horizon material (site PJ, Fig. 3) had a significant time × water interaction. In all three of these cases, however, the timing of the maximum C flush was unaffected by soil water content.

ANCOVA was used to test the effect of soil water content on 5 d C and N flush values, with the corresponding 1 d flush values as covariates. These analyses allowed us to determine if the relationships between 1 and 5 d flushes were altered by soil water content. We found that water content did not affect the relationship between 1 and 5 d flushes of both C and N in samples taken from O horizons ($F = 3.232$, $P = 0.077$ for C and $F = 0.001$, $P = 0.975$ for N; data not shown). This was also true for mineral soils taken from A horizons ($F = 1.768$, $P = 0.189$ for C and $F = 0.024$, $P = 0.877$ for N; data not shown).

3.2. Effects of fumigation time and soil water content on C/N ratio of flush

In several cases, fumigation time had a significant effect on the C/N ratio of the fumigation flush both for samples

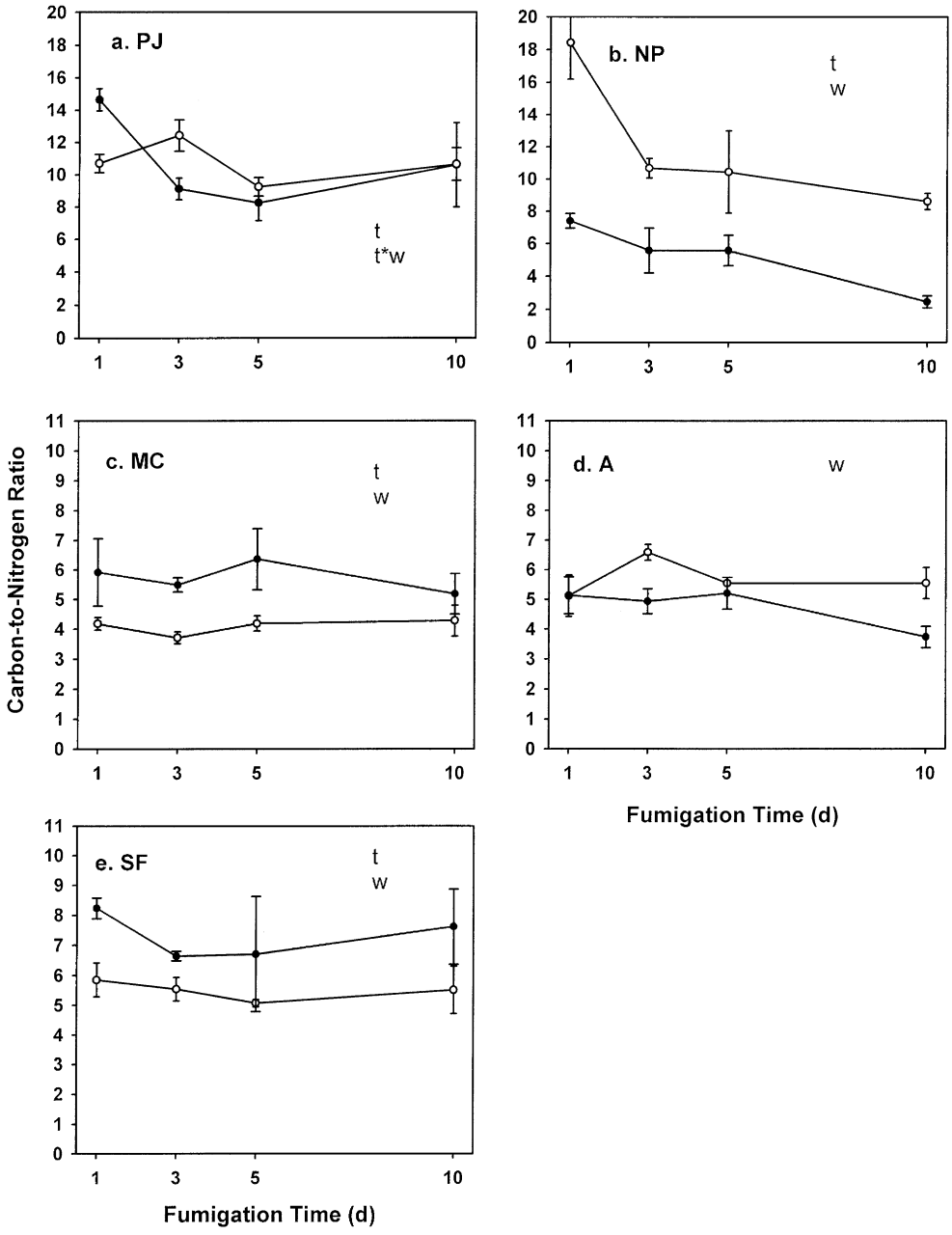

Fig. 7. Extractable carbon-to-nitrogen (C/N) ratios in top 15 cm of mineral soil (A horizon) taken from New Mexico sites after 1, 3, 5, and 10 d of exposure to chloroform. Each data point (● = field capacity; ○ = field-moist) represents the mean of three replicates ± one SEM. Letters in figure indicate whether time (t), water (w), or time × water ($t \times w$) were significant ($p < 0.05$); ns = not significant. Order of panels follows elevational gradient from lowest (Pinyon–Juniper) to highest site (Spruce–Fir). See Table 1 for key to site labels.

taken from O horizons (sites PJ-p, PJ-j, NP, OP, DF, and RD; Figs. 5 and 6) and A horizons (sites PJ, NP, SF, OP, MH, RD, HS; Figs. 7 and 8). Generally for these soils, the C/N ratio of the fumigation flush from O horizon materials increased with the length of time exposed to $CHCl_3$, while the C/N ratio of fumigation flushes decreased over time in A horizon soils. The relative magnitude of the change in C/N ratios of fumigation flushes resulting from different times of exposure to $CHCl_3$ ranged from about 1 to 10 kg C kg^{-1} N in soils taken from both O and A horizons.

Soil water content also had a significant, albeit small, effect on the C/N ratio of the fumigation flush in a majority of the O horizon samples (sites PJ-p, PJ-j, MC, SF, OP, MH, DF, and RD; Figs. 5 and 6) and A horizon samples (sites NP, MC, A, SF, OP, MH, RD, HS; Figs. 7 and 8). However, we found no consistent pattern between the direction of the change in the C/N ratio of the flush and the change in soil water content for these soils. Additionally, several samples from O horizons (sites NP, OP, and RD; Figs. 5 and 6) and A horizons (sites PJ, OP,

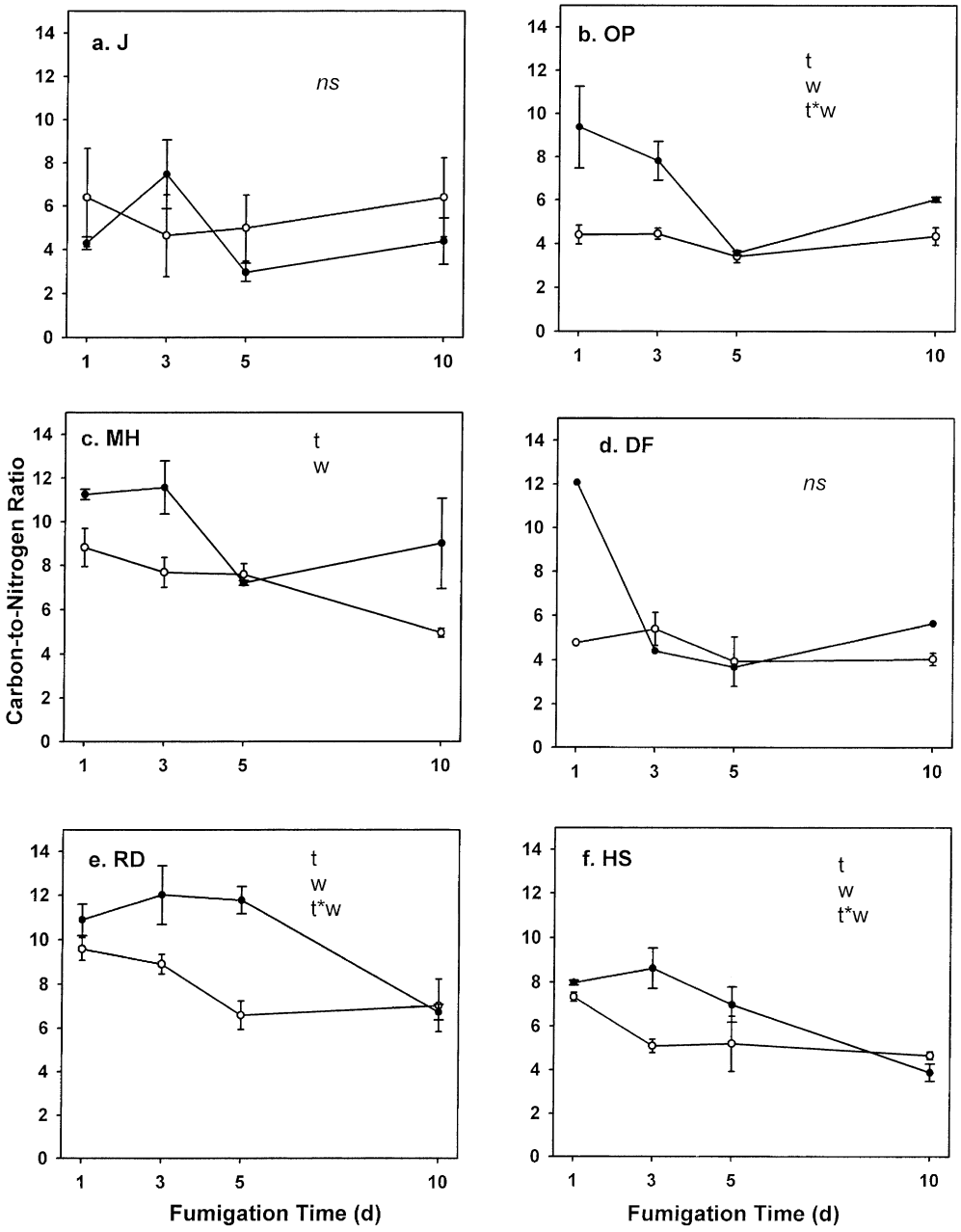

Fig. 8. Extractable carbon-to-nitrogen (C/N) ratios in top 15 cm of mineral soil (A horizon) taken from Oregon sites after 1, 3, 5, and 10 d of exposure to chloroform. Each data point (● = field capacity; ○ = field-moist) represents the mean of three replicates ± one SEM. Letters in figure indicate whether time (t), water (w), or time × water ($t \times w$) were significant ($p < 0.05$); ns = not significant. Order of panels follows longitudinal gradient from most interior site (Juniper) to coastal site (Western hemlock/Sitka spruce). See Table 1 for key to site labels.

RD, and HS; Figs. 7 and 8) had significant time × water interaction terms.

4. Discussion

Our results demonstrate that increasing the $CHCl_3$ fumigation period of the FE method alters the magnitude of both C and N flushes in many (but not all) soils. Across all the forest floor materials and water contents tested, using a 1 d $CHCl_3$-exposure, on average, underestimated the maximum fumigation C and N flush by 24 and 18%, respectively. Similar relative errors occurred in the mineral soils. While this relative error may be acceptable for assessing large changes in microbial biomass in a given soil type, individual soils had 1 d flushes that underestimated C and N flushes by as much as 58 and 79%, respectively. Such large relative errors suggest that the influence of fumigation time on flush values should be evaluated when comparing microbial biomass nutrient pools across contrasting soils.

We are aware of only one other study that examined the response of both C and N in the same soil to variable exposure time to $CHCl_3$. Ross and Tate (1993) found in a strongly acid southern beech soil that increasing exposure time from 1 to 5 d increased extractable C values in the organic (F + H layers) horizon, but either had no effect (0–8 cm) or decreased (8–20 cm) the C flush in the mineral soil. In contrast, increasing exposure time had no effect on extractable N in the organic horizon, but increased N flushes in mineral soil horizons. Davidson et al. (1989) found that 1 d exposure was adequate to achieve a maximum N flush in a forest mineral soil, but 5–7 d were required in a similar soil from an annual grassland. Brookes et al. (1985a) reported that prolonging exposure of grassland and arable soils to $CHCl_3$ increased the amount of total N released from 1 to 5 d, after which the total amount released reached a plateau. These studies, taken together, suggest that the effect of exposure time to $CHCl_3$ vapor on the extractability of C and N is highly variable among soil types.

The question of the appropriate length of exposure of a soil to $CHCl_3$ is critical to measurements of microbial biomass C and N using the FE method because most researchers assume that a constant proportion of the maximum flushes can be obtained after a 1 d exposure, and that maximum flushes occur after 5 d (Brookes et al., 1985b). We found that, across all sites, C and N flushes after 1 and 5 d in soils taken from a given horizon were highly correlated, and had slopes near one. However, the ratio of 1–5 d flush values varied dramatically among soils. In O horizons, this ratio ranged between 0.65 and 1.4 for C flushes, and between 0.63 and 2.0 for N flushes. Similarly, this ratio ranged from 0.74 to 1.4 for C flushes, and from 0.21 to 1.4 for N flushes in A horizons.

Variability in the ratio of 1–5 d C and N flushes are also noted in the literature. For instance, Brookes et al. (1985b) reported an average 1–5 d N-flush ratio of 0.78 for two arable soils. Davidson et al. (1989) found a N-flush ratio of about 1.0 for a forest soil, while a ratio of only 0.57 for a grassland soil. Ross and Tate (1993) reported 1–5 d N-flush ratios of 0.92 for F + H layers and 0.86–0.88 for mineral soils from a southern beech forest. They also reported 1–5 d C-flush ratios of 0.84 and 1.0–1.1 for these same horizons, respectively.

We also found frequently that 5 d of $CHCl_3$ exposure did not result in maximum C of N flushes. Davidson et al. (1989) reached this same conclusion for the mineral soil of annual grassland in California. From these studies, it is clear that assuming that a certain fraction of the maximum C or N flush can be obtained after a 1 d fumigation can result in either underestimates or overestimates of the actual maximal C and N flushes, leading to erroneous measures of microbial biomass C and N.

We found that soil water content strongly affected C and N flushes, consistent with the results of several other studies and the conclusion that appropriate soil water content selection is critical for estimating microbial biomass C and N by the FE method (Davidson et al., 1989; Ross, 1989; Sparling and West, 1989; Sparling et al., 1990; Badalucco et al., 1997). However, the ANCOVA results indicated that across all soils, the relationship between C and N flushes after 1 and 5 d was unaffected by changes in water content, even though the absolute and relative amount of water added to a given soil differed substantially. This suggests that soil water content does not have a strong effect on the time response of the fumigation flush (i.e., how the fumigation flush changes over a 10 d time course) when evaluated across a broad range in soil types.

Although we observed statistically significant effects of water on C and N flushes in the majority of the 23 soils we tested, organic materials (O horizons) showed considerable variation in the directionality of their response to increased water content. O horizons that were more fibrous (mor-type) and drier at the field-moist state tended to show higher C and N flushes with increasing water content (site PJ-p, -j; Fig. 1a and b), while less fibrous (mull-type) horizons that were wetter at the field-moist state tended to exhibit lower C and N flushes with increasing water content (sites A and RD; Figs. 1e and 2e). In these mull O horizons, diffusion of $CHCl_3$ vapor may be reduced in materials at field capacity water contents, thereby protecting some cells from lysis (Davidson et al., 1989; Badalucco et al., 1997). We know of no other studies that have systematically assessed fumigation responses to changes in soil water content in O horizons, so we are uncertain of the generality of this pattern. However, Couteaux et al. (1990) hypothesized that the high water contents of the F and H layers from a mixed forest with a moder-type O horizon protected protozoan cells from lysis because of the low solubility of $CHCl_3$ in water. In contrast, Sparling et al. (1990) suggested that low water contents of organic soils resulted in low C flushes; they based this conclusion on the higher C flushes obtained with these same soils measured at higher water contents but in different years. They recommended fumigating organic and inorganic soils at a matric potential of -5 kPa, a higher water content than that of any of the soils used in our study.

In mineral soils, the more common response of C and N flushes to increases in soil water content is increases in C and N flushes (Davidson et al., 1989; Ross, 1989; Sparling and West, 1989; Sparling et al., 1990), as we generally found for the mineral soils we studied. Brookes et al. (1985a) suggested that water enhances the processes that release soluble N by $CHCl_3$ (e.g. extracellular enzymes and solubilization of cell wall material).

We also found some evidence that timing of the maximum flush of N (but not C) depends on soil water content. Where there was an interaction of soil water content and time of exposure to $CHCl_3$, wetter soils tended to exhibit maximum flushes of N after longer exposure to $CHCl_3$. This result is consistent with the hypothesis discussed earlier that $CHCl_3$ diffusion and effective solubilization of microbial N is inhibited in some cases by water held in soil micropores (i.e. those retaining water at -33 kPa; Brady and Weil, 1999).

Changes in the C/N ratio of the fumigation flush over time, and its sensitivity to changes in soil water content, suggest that fumigation conditions impact the nature of the organic pools extracted using the FE method. However, the general increases in the C/N ratio of the fumigation flush in organic horizons and decreases in the C/N ratio of the flush in mineral horizons with increasing exposure to $CHCl_3$ also may simply reflect the relative dominance of fungal (relatively high C/N ratio) and bacterial (relatively low C/N ratio) biomass, respectively, in these different horizons. As the fumigation time increases, the C/N ratio of the flush may become increasingly representative of the dominant soil microbial functional group present. If this hypothesis is correct, we would expect a plateau to occur in the C/N ratio of the flush; however, such a plateau was not frequently found (Figs. 7 and 8), suggesting that the relative contribution of non-biomass soil organic matter pools to the total flush may change during fumigation exposure.

Very few investigators have assessed the impact of fumigation conditions on the C/N ratio of the fumigation flush. Ross and Tate (1993) found that fumigation time from 1 to 5 d had little effect on the C/N ratio of the flush produced from organic and mineral horizons from a southern beech forest (varying by <2 kg C kg^{-1} N). For a wide range of pasture mineral soils from New Zealand, Sparling and West (1989) reported that the C/N ratio of the fumigation flush (with overnight fumigation) changed dramatically between air-dry soils and soils rewetted to 20 or 50% gravimetric water content. In our study, we found a similar range of changes in the C/N ratio of the flush from smaller changes in water content (0–11 kg C kg^{-1} N after a 1 d fumigation). However, unlike the present study, Sparling and West (1989) found that soils at a higher water content almost always had a lower C/N ratio of the fumigation flush. Their water treatment consisted of an extreme and consistent change in water content while ours was more modest and variable, which may have added to the variability in the fumigation response pattern we found.

Our results, and the limited data reported by others (Sparling and West, 1989; Martikinen and Palojärvi, 1990), suggest that great care must be taken in using the C/N ratio of the fumigation flush to suggest the relative dominance of fungi and bacteria within soils. Using a 1 d flush to estimate the C/N ratio of the microbial biomass underestimated the C/N ratio based on maximum C and N release by as much as 23%, and overestimated it by as much as 66%, across all the forest floor materials tested in our study. Additionally, the range in relative error of the estimated microbial C/N ratio in mineral soils based on a 1 d $CHCl_3$ exposure was about twice that of forest floor material. These results may help explain why some simulation models have failed to predict C and N mineralization based on C/N ratios of the microbial biomass generated from the FE method (Dendooven et al., 2000).

Our conclusions concerning the effects of $CHCl_3$ exposure time and soil water contents on C and N flushes from the FE method follow; many are similar to those reported by Davidson et al. (1989) for N flushes measured on a much smaller set of soils using the FE method:

1. The appropriate duration of fumigation for FE differs across contrasting soil types, but in general half the observed soils required only 1 d fumigations, and O horizon soils required longer fumigation times compared to A horizon soils.
2. Increasing water content generally resulted in greater maximum C and N flushes but did not affect the timing of those maxima. For comparative work across soil types, we strongly recommend that the influence of water content on fumigation flushes be assessed for each soil type before using the FE method to make generalizations about differences in the sizes of microbial pools.
3. Use of a 1 d fumigation to measure $CHCl_3$-labile C and N pools and applying a correction factor (i.e. k_c and k_n) to adjust these values to the maximum C and N flushes is not recommended unless the consistency of the correction factors has been previously assessed for that soil.
4. More work (perhaps using isotopically labeled microbial biomass) needs to be conducted on how the nature of the organic pools extracted from the soil changes with varying time of exposure to $CHCl_3$ vapor.
5. Given the variability in the response of the C/N ratio of the fumigation flush to changes in fumigation conditions (i.e. fumigation time and water content), we recommend that these ratios not be used to infer differences in the composition of the microflora among soils without first rigorously assessing how these factors affect C and N fumigation flushes from the individual soils.

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