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Stable-Isotope Analysis of a Combined Nitrification-Denitrification Sustained by Thermophilic Methanotrophs under Low-Oxygen Conditions

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To simulate growth conditions experienced by microbiota at O_2-limited interfaces of organic matter in compost, an experimental system capable of maintaining dual limitations of oxygen and carbon for extended periods, i.e., a pO_2-auxostat, has been used. ^{15}N tracer studies on thermophilic (53°C) decomposition processes occurring in manure-straw aggregates showed the emission of dinitrogen gas from the reactor as a result of simultaneous nitrification and denitrification at low pO_2 values (0.1 to 2.0%, vol/vol). The N loss was confirmed by nitrogen budget studies of the system. Depending on the imposed pO_2, 0.6 to 1.4 mmol of N/day (i.e., 20 to 40% of input N) was emitted as N_2. When the pO_2 was raised, the rates of both nitrification and denitrification increased instantaneously, indicating that ammonia oxidation was limited by oxygen. In auxostats permanently running at $pO_2 \geq 2\%$ (vol/vol), the free ammonium pool was almost completely oxidized and was converted to nitrite plus nitrate and N_2 gas. Labelling of the auxostat with [^{13}C]carbonate was conducted to reveal whether nitrification was of autotrophic or heterotrophic origin. Incorporation of $^{13}CO_2$ into population-specific cellular compounds was evaluated by profiling the saponifiable phospholipid fatty acids (FAs) by using capillary gas chromatography and subsequently analyzing the $^{13}C/^{12}C$ ratios of the individual FAs, after their combustion to CO_2, by isotope ratio mass spectrometry. Apart from the observed label incorporation into FAs originating from a microflora belonging to the genus *Methylococcus* (type X group), supporting nitrification of a methylotrophic nature, this analysis also corroborated the absence of truly autotrophic nitrifying populations. Nevertheless, the extent to which ammonia oxidation continued to exist in this thermophilic community suggested that a major energy gain could be associated with it.

Much of the early microbiological research on composting has been limited to the determination of taxonomic diversity and cell numbers in the mesophilic and thermophilic stages of the process (for a review, see reference 10). In more recent studies, attempts were made to relate microbial activity to temperature, biomass (27, 28), and specific groups of organisms (34, 35, 48). Regardless of the objectives of these studies, the microbial decomposition of organics during composting has been generally considered a predominantly aerobic process. In situ measurements in macropores of well-aerated compost, however, have shown that the oxygen partial pressure (pO_2) readily drops below 10%, often down to 2.5%, air saturation (38, 49). Hence, the growth of aerobic microbes in the boundary layer of compost aggregates and the free air space of pores is likely to be limited by oxygen diffusion. Oxygen limitation, which is caused by a reduced solubility of oxygen at the elevated temperatures present during composting, is probably the rule rather than the exception throughout the decomposition process. Similar to anoxic habitats in otherwise oxic environments such as the oxygen-depleted microniches present in marine snow or fecal pellets (2), the interior of organic-rich compost solids will be devoid of oxygen if the diameter of the aggregates exceeds a few millimeters (20). Indeed, in the preparation of mushroom compost, a substantial and continuous formation of methane has been shown to occur during all stages of the process (12), and, remarkably, samples taken from the outside of compost stacks produced amounts of methane comparable to samples taken from the inside. Low-oxygen habitats easily give rise to a strong coupling of aerobic and anaerobic metabolism (17), and oxic-anoxic interfaces are observed to coincide with sites of enhanced microbial activity and element cycling in many natural systems (39, 46, 50). To date, other environments than the compost stack have attracted attention in studies on the ecophysiology of microbes at oxic-anoxic interfaces. Despite the technical and practical constraints on experimental research posed by its texture, compost represents an interesting habitat for microbial studies on low-oxygen physiology because of its potential relevance to the worldwide practice of composting.

Because of the heterogeneity of compost with respect to conditions and composition, an experimental system has been developed that is capable of maintaining O_2 limitation in slurries of organic solids for extended periods at a fixed average degree of mineralization of the substrates: a pO_2 auxostat (37). At 53°C and a pO_2 of 0.1% (vol/vol) in the reactor slurry consisting of manure and straw, 10 to 20% of the mineralization proceeded strictly anaerobically. The CH_4 formed, however, was largely oxidized by methanotrophs. Quite unexpectedly, a substantial nitrogen loss from the reactor occurred (≈20% of influent N) as a result of ammonium oxidation and concurrent denitrification with dinitrogen gas as the end product (37).

In composting, nitrification is considered a marginal process,

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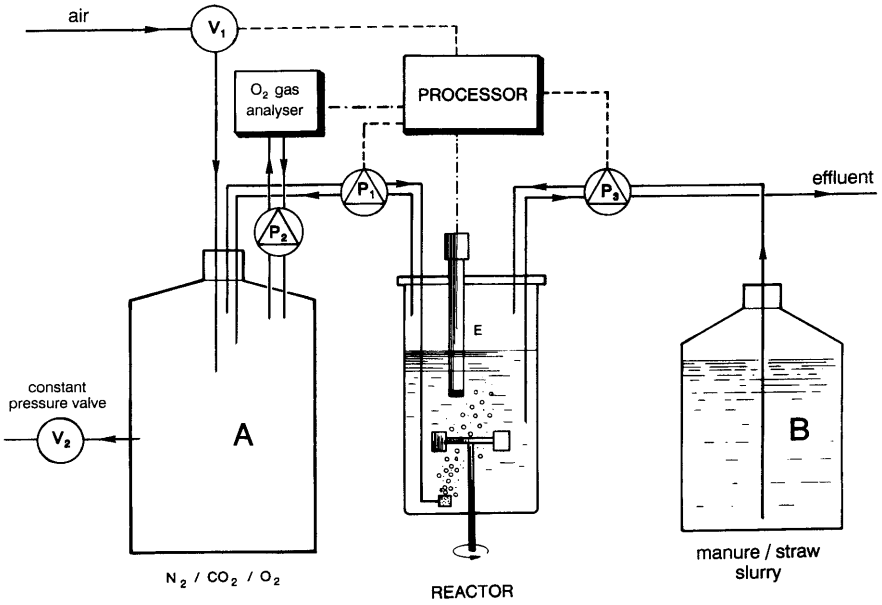

FIG. 1. Schematic representation of pO_2 auxostat culture device. A, gas reservoir; B, manure-straw slurry reservoir; E, oxygen electrode; P_1 and P_2, gas recirculation pumps; P_3, influent/effluent pump; V_1, solid-state valve of flow meter; V_2, barometric valve.

related to heterotrophic microbial activity (1, 11), since autotrophic nitrifiers apparently are unable to thrive at (or adapt to) the operational temperatures (50 to 60°C) attained in the thermophilic stage (16). N loss in composting is thought to be due primarily to the volatilization of ammonia. However, whether biological processes contribute to the loss (apart from producing the ammonia) has, to our knowledge, never been verified by means of a rigorous method such as ^{15}N labelling. In this communication, we will characterize N mineralization processes in our model in more detail by using stable isotope labelling. The quantitative importance of a nonautotrophic nitrification at 53°C, and the direct dependence of this activity on imposed oxygen levels in the reactor will be demonstrated. A lipid biomarker analysis of membrane-derived fatty acids in conjunction with ^{13}C label incorporation from [^{13}C]carbonate was conducted to reveal the identity of the microbiota involved in the observed nitrification.

MATERIALS AND METHODS

pO_2 auxostat and control system. Figure 1 depicts a scheme of the experimental system applied. Essentially, a pO_2 auxostat is a continuous-culture device similar to a turbidostat. However, instead of controlling the cell density of the culture by means of a turbidity sensor, biomass in the reactor is indirectly controlled by monitoring the amount of oxygen consumed from an air reservoir. To this end, gas from reservoir A in Fig. 1 is continuously recirculated through the reactor slurry and an oxygen gas analyzer (Servomex) by pumps P_1 and P_2, respectively. Below a given pO_2 value in reservoir A (pO_2^A), a processor/control unit (Biocontroller ADI 1020; Applicon) will simultaneously activate a peristaltic medium/effluent pump (P_3) and a solid-state relay valve (V_1) of an air flow meter (Brooks). The ratio of the amount of air to slurry supplied to reservoir A and the reactor, respectively, is similar to the ratio of the volume of this reservoir over the reactor contents (namely, 23:1.3). Excess gas leaves reservoir A via barometric precision valve V_2 (Bühler), keeping the absolute pressure in the system at $(1{,}040 \pm 0.6) \times 10^5$ mPa. Changes in the dilution rate (D) of the system are achieved by adjusting the pO_2^A set value.

The oxygen partial pressure in the fluid of the reactor slurry (pO_2^R) is maintained by regulating the rate of gas recirculation by P_1 with a Clark-type oxygen electrode (Ingold) connected to the bioprocessor. The temperature of the reactor was maintained at 53°C, and the pH was automatically titrated at 6.8 to 7.0. More details on the performance of the pO_2 auxostat are given in reference 37.

Manure-straw substrate. The composition of the feed was 2.3 g of dry poultry manure plus 2 g of chopped wheat straw (1 to 4 mm in length) in 1 liter of tap water. Microbial growth in the feed reservoir was prevented by acidifying the contents with HCl to pH 2.5 and maintaining 100% N_2 in the headspace of the vessel. The manure-straw mixture was kept in suspension by agitation. To ensure a feed of constant quality throughout the experiments, 1-day-old manure was freeze-dried, ground, and stored at −20°C in closed bottles before use. The approximate composition of fresh poultry manure with respect to minerals, major organic fractions, and the distribution of nitrogen species is as follows (data from references 4 and 52; mineral N and ash data from this study). The mineral composition (as a percentage of dry matter) is 4.9% N, 2.26% P, 1.89% K, 6.69% G, 0.75% Mg, 0.40% Na, 0.73% S, and 31% ash. The major organic fractions (as a percentage of dry matter) are 65% neutral detergent-soluble material, 17% hemicellulose, 15% cellulose, 3% lignin, 8% protein, 8% uric acid, and 4% lipid. The range of nitrogen species (as a percentage of total N) is 25 to 28% amino acid (protein), 4 to 8% urea, 8 to 12% ammonium, 48 to 61% uric acid, and 1 to 7% other.

Inoculum. The reactor was inoculated with material from an approximately 2-month-old compost pile set up with poultry manure and wheat straw.

Tests on autotrophic nitrification. Coupled nitrification-denitrification was studied in the presence of inhibitors of autotrophic nitrification by using subcultures from the auxostat. To this end, 17-ml screw-cap Hungate tubes containing freeze-dried manure and chopped straw were gamma sterilized (6-megarad dose) and subsequently reconstituted by applying 6 ml of sterile water to obtain the medium composition used in the auxostat. Each tube received filter-sterilized (pore size, 0.2 μm) [^{15}N]ammonium (atom% ^{15}N, >99%; concentration, 0.5 mM), and an appropriate amount of one of the following inhibitors (except the controls): dicyandiamide, 90 μM; chlorate, 10 mM; and acetylene, 10 μM. Reactor slurry was serially diluted (1:10) into the tubes up to a 10^{-10} dilution. The tubes were incubated at 53°C in a horizontal position on a rotary shaker (40 rpm) or statically for 35 days. Headspaces were sampled for $^{15}N_2$ analysis at $t = 2$ days and subsequently every 6 to 7 days by replacing 5 ml with sterile air. Culture fluid (0.2 ml) from the tubes was immediately analyzed for nitrite and nitrate.

Enrichment cultures for autotrophic nitrifiers were set up and analyzed as described above. However, instead of the sterile manure-straw medium, filter-sterilized effluent from the auxostat was used.

Analytical methods. Gas species present in reservoir A (CO_2, O_2, CH_4, and N_2O) were analyzed on a Packard gas chromatograph with a katharometer detector (1.5-m packed Porapak Q column at 50°C; 15-ml/min N_2 carrier flow).

Nitrate and nitrite were determined by high-performance liquid chromatography analyses with UV detection at 205 nm as described previously (18). Ammonia was determined with L-glutamate dehydrogenase by using a Sigma test kit as specified by the manufacturer.

Microbial biomass was measured spectrophotometrically by means of a micro-Lowry method with a Sigma test kit. Protein was extracted from samples by ultrasonic disruption of cells in the presence of sodium dodecyl sulfate. After six 30-s periods of sonication with 30-s intervals, the mixture was centrifuged at 40 × g for 10 min. Protein in the supernatant was precipitated with trichloroacetic acid in the presence of deoxycholate as specified by the Sigma protocol and measured. Solubilization of cell protein by boiling in 1 N NaOH was abandoned,

since this procedure released substances from the straw fraction that interfered with the Lowry determinations.

Total nitrogen of the influent and effluent of the reactor was determined by Kjelldahl destruction in triplicate by standard methods.

Lipid-derived FA analyses. Cellular fatty acids (FA) were extracted with methanolic NaOH from pelleted reactor samples containing approximately 40 to 80 mg (wet weight) of cells and subsequently methylated as specified by the protocol supplied with the automated Microbial Identification System (Microbial ID, Newark, Del.). FA methyl esters (FAMEs) were extracted into hexane–methyl-*tert* butyl ether, separated by capillary gas chromatography (GC), and named by using the Microbial Identification System FAME identification software. Structural confirmation of most of the FAMEs in the peak library data base has been accomplished by mass spectrometry (44). A further verification of the identity of the FAMEs was according to their equivalent chain length by comparing elution times with retention time data obtained from authentic FAME standards on both a nonpolar (Ultra 2; 50-m) and a polar (BPX 70; 50-m) column.

^{15}N-labelling experiments. Denitrification and coupled nitrification-denitrification were measured by quantifying the evolution of $^{15}N_2$ in the headspace after spiking the reactor fluid with $^{15}NO_3^-$ and $^{15}NH_4^+$, respectively. The sensitivity of $^{15}N_2$ analysis was increased by reducing the total headspace volume (reactor plus reservoir A) to approximately 1,000 ml. The pO_2 in this headspace volume was reduced approximately threefold to increase the rate of gas recirculation by P_1. In this way N_2 species in the liquid and gas phase equilibrated within a few minutes. Denitrification under anoxic conditions was studied with pure N_2 in the headspace and gas recirculation by P_1 maintained at a fixed rate.

In the $^{15}NO_3^-$ amendment experiments, the amount of N_2 in the sample attributable to denitrification was calculated by the method of Mulvaney (31) from measured values of $r = {}^{29}N_2/{}^{28}N_2$ and $r'' = {}^{30}N_2/{}^{28}N_2$, with atmospheric N_2 (0.3663 atom% ^{15}N) as the reference gas. The mole fractions of $^{29}N_2$ and $^{30}N_2$ evolved can be expressed in terms of the mole fraction of ^{15}N in the nitrate denitrified ($^{15}X_N$), since ^{14}N and ^{15}N are randomly distributed among the N_2 molecules produced:

$$ {}^{29}N_{2\,ev}/\text{total } N_{2\,ev} = 2\,{}^{15}X_N(1 - {}^{15}X_N) \quad (1) $$

and

$$ {}^{30}N_{2\,ev}/\text{total } N_{2\,ev} = ({}^{15}X_N)^2 \quad (2) $$

Dividing equation 2 by equation 1 gives

$$ {}^{30}N_{2\,ev}/{}^{29}N_{2\,ev} = {}^{15}X_N/2(1 - {}^{15}X_N) \quad (3) $$

By using appropriate approximations, $^{30}N_{2\,ev}$ and $^{29}N_{2\,ev}$ can be calculated from r and r'' within a few percent of the actual values. For a detailed treatise of the derivation of the original formulas, the reader is referred to papers by Mulvaney (31) and Mulvaney and Boast (32).

The $^{30}N_{2\,ev}/^{29}N_{2\,ev}$ ratio was used for assessing the rate of nitrification by combining equations 3 and 1 (or 2). The rate was calculated as the total amount of N evolving by denitrification (total $N_{2\,ev}$) minus the nitrate N disappearing from the nitrate pool in the same period. Dissimilatory reduction of NO_3^- to NH_4^+ (nitrate ammonification) was included in the calculation of the nitrification rate, as follows:

$$ dN/dt_{\text{nitrification}} = (dN/dt_{\text{denitrification}} + dN/dt_{\text{ammonification}}) - dN/dt_{\text{nitrate pool}} \quad (4) $$

Provided that data on the recovery of the ^{15}N label in both the headspace and the ammonium pool of the culture are available, $dN/dt_{\text{ammonification}}$ can be inferred from $dN/dt_{\text{denitrification}}$.

The rate of N_2 production in the $^{15}NH_4^+$ amendment experiments was calculated from the ratio $^{14}N/^{15}N$ of the enriched ammonium pool and the linear increase of $^{15}N_2$ in the headspace during the first 4 to 6 h following the addition of the label.

Nitrate ammonification was determined by measuring the enrichment in ^{15}N of the NH_4^+ pool after spiking the reactor with $^{15}NO_3^-$. Direct immobilization of NO_3^- into the organic N pool (microbial biomass) was not determined, because this process was considered negligible due to the presence of NH_4^+ far in excess of the needs for microbial growth.

^{15}N-labeled nitrous oxide was determined as described by Mulvaney and Kurtz (33).

Analysis of ^{15}N fractions. For determination of $^{15}N_2$-enrichments, 4-ml gas samples from the headspace of the auxostat were introduced directly via a syringe into the injection port of a SIRA series II isotope ratio mass spectrometer (IRMS) (VG) equipped with a dual microinlet. The gas samples were injected over magnesium perchlorate and Ascarite (Sigma) to eliminate H_2O vapor and carbon dioxide, respectively.

$^{15}NH_4^+$ enrichments were measured after extraction of ammonium into boric acid by an alkaline distillation. The distillates were evaporated to dryness on a boiling-water bath and subsequently converted into N_2 by Dumas combustion with a Carlo Erba 1500 CN elemental analyzer. The resulting N_2 was introduced into the IRMS via a continuous-flow interface (CF-IRMS). Distillate samples were measured in duplicate with urea (atom% ^{15}N = 0.3678) as an external standard. The precision of the dual-inlet and CF-IRMS ^{15}N measurements was better than 0.0001 atom%.

^{13}C-labelling experiments. Because of the low growth yield of autotrophic populations, CO_2 fixation by these populations could be easily lost in the bulk of biomass derived from organotrophic populations incorporating CO_2 by anapleurotic routes (e.g., phosphoenolpyruvate-carboxylase activity). Therefore, the incorporation of $^{13}CO_2$ into separate phospholipid-derived fatty acids has been monitored by GC-combustion-IRMS analyses. This type of analysis offers two distinct advantages. (i) Anapleurotically fixed CO_2 is not funneled toward biosynthetic routes of straight-chain or cyclopropane FAs (branched FAs may contain some label, since C_4 and C_5 trichloroacetic acid intermediates act, though indirectly, as primers in biosynthesis [45]). (ii) Most microbial species contain a variety of FAs, so that the chance of encountering an FA not shared or only partly shared by other members of the community is a real one, providing more resolution in the analysis. By a continuous labelling with [^{13}C]carbonate, the CO_2-HCO_3^- system of the auxostat was elevated by 0.2 atom%. [^{13}C]carbonate was delivered to the reactor as the sodium salt in synchrony with the feed by a separate peristaltic pump. CO_2 incorporation into microbial biomass was evaluated by measuring ^{13}C enrichment of cellular protein and lipid-derived FAs after their combustion to CO_2 (see below).

The isotopic composition was expressed in δ^{13}C values (parts per thousand difference from a standard): $\delta^{13}C = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$, where R is the $^{13}C/^{12}C$ ratio and the standard is PeeDee Belemite ($^{13}C/^{12}C_{PDB} = 0.0112372$).

Analysis of ^{13}C fractions. Microbial protein was extracted by sonication of cells as described above, except that sodium dodecyl sulfate was omitted and concentrated HCl was used instead of trichloroacetic acid and deoxycholate in the precipitation. Precipitates were washed twice with distilled water, freeze-dried, and loaded onto the Carlo Erba analyzer for determination of ^{13}C enrichment by CF-IRMS with pepsine ($\delta^{13}C = -22.36‰$) as an external standard. The δ^{13}C of manure was determined after removal of inorganic carbon by acidifying slurries with HCl and subsequent freeze-drying.

^{13}C enrichment of lipid-derived FAMEs was determined by separating the saponifiable components of a whole-culture lipid extract by capillary GC and a subsequent $^{13}C/^{12}C$ ratio analysis of the individual FAMEs after their on-line combustion to CO_2 (GC-combustion-IRMS; Delta S, Finnigan MAT; Bremen, Germany). Baseline separation of most of the FAME peaks was achieved with a 50-m Ultra 2 column (0.32 mm [inner diameter] by 0.5 μm film; HP 123435B-115). Each peak was integrated over its entire width, and the ^{13}C data were corrected for background. The precision of the δ^{13}C measurements is better than 0.4%.

RESULTS

pO_2 auxostat. Throughout the nitrification-denitrification studies, the average degree of mineralization of the manure-straw slurry in the reactor was kept constant. Because a major part of the organics present in the feed consisted of recalcitrant lignocellulose fibers, the growth of the microflora was essentially carbon limited as indicated by the immediate enhanced respiratory activity following addition of easily degradable substrates, such as acetate or ethanol, to the reactor fluid. In Table 1, some culture parameters have been summarized typically obtained in the auxostat culture maintained at $pO_2^R = 0.1\%$ (vol/vol).

As determined by confocal laser scanning microscopy (6), filamentous populations represented a major part of the microbial biomass (approximately 30%). At least four morphologically different filamentous populations could be distinguished: a fungal one, branching actinomycetes, and species probably belonging to the genus *Flexibacter* or *Cytophaga*.

^{15}N labelling. [^{15}N]nitrate-labelling experiments were conducted, imposing four different aeration regimes: apart from the steady-state value of 0.1, the pO_2^R of the culture was also brought temporarily to 0.0, 0.5, and 2.0% (vol/vol). As a direct result of aeration shift-ups, the reactor fluid concentration of nitrate plus nitrite was observed to increase within a few hours from ≈2 μM at $pO_2^R = 0.1\%$ to approximately 5 and 10 μM at $pO_2^R = 0.5$ and 2%, respectively (data not shown).

After spiking the reactor fluid with [^{15}N]nitrate (±65 μmol; ^{15}N atom%, >99.7), the apparent rate of nitrate consumption decreased with increasing pO_2^R (Fig. 2). However, analyses of the N_2 species present in the headspaces showed increasing amounts of $^{29}N_2$ evolved at increasing pO_2^R, whereas at $pO_2^R = 0.0\%$, >93% of the emitted ^{15}N label evolved as $^{30}N_2$. These observations indicated an enlarged availability of [^{14}N]nitrate

TABLE 1. Auxostat culture parameters at pO_2^R = 0.1% after 1 year of operation[a]

Parameter	Value in:			
	Influent	Effluent	Air in	Headspace
Kjeldahl-N (mM)	7.9 ± 0.4	6.4 ± 0.2		
Ammonium (mM)	0.7–0.8	2.4–2.6		
Nitrate (μM)	60–70	2–3		
Nitrite (μM)	0–10	<2		
Number of cells (10^8/ml)	ND[b]	5–9 (30)[c]		
Total protein (mg/liter)	120	190		
Methane (μM)[d]		0.3–0.7		
Oxygen (%, vol/vol)			20.9	18.6–18.8
Carbon dioxide (%, vol/vol)			0.03	1.9–2.1
Methane (%, vol/vol)				0.03–0.07
Nitrous oxide (%, vol/vol)				Trace[e]

[a] D = 0.34 ± 0.04 day^{-1}.
[b] ND, not determined.
[c] The percentage of filamentous microorganisms determined by the method of Bloem et al. (6) is shown in parentheses.
[d] Calculated from the percentage of CH_4 in the head space (53).
[e] Detectable only after reduction to $^{15}N_2$ in labelling experiments.

for denitrification at higher oxygen levels in the reactor. Indeed, the continuous formation of [^{14}N]nitrate in the aerated systems was reflected by a steadily decreasing $^{15}X_N$ value of the nitrate pool following the addition of the ^{15}N label (although $^{15}X_N$ always was observed to rise transiently just after spiking [Fig. 3]). Assuming sufficient isotopic uniformity of the nitrate pool 2 h after spiking, the $^{30}N_2^{ev}/^{29}N_2^{ev}$ ratio was used to assess the rate of nitrification at each pO_2^R by using equation 4. In the determination of total N_2^{ev}, the formation of N_2O was neglected since the ^{15}N label in this product accounted for less than 1‰ of total ^{15}N applied at all pO_2^R. However, nitrate ammonification depended on the pO_2^R imposed (Fig. 4) and was taken into account accordingly. Thus, a strong increase of the nitrification rate was apparent with increasing levels of oxygen in the reactor (Fig. 5).

At pO_2^R = 0.1% N, loss from the reactor was also evaluated by [^{15}N]ammonium labelling and by comparing the total N-Kjeldahl of reactor input and output. Both methods yielded similar rates of N loss (≈660 ± 55 μmol of N · day^{-1}), approximately sixfold higher than indicated by [^{15}N]nitrate labelling (95 ± 9 μmol N · day^{-1}). As shown by Vanden Heuvel et al. (51), the evolution of N_2 is seriously underestimated by the latter technique if the nitrate denitrified does exist in multiple pools differing in isotopic composition. Therefore, a shift-up/shift-down experiment (pO_2^R, 0.1 → 2.0 → 0.1% [vol/vol]) has been conducted to check the assumption of label uniformity. In Fig. 6, the apparent $^{15}X_N$ of the nitrate pool is shown before and after the pO_2^R was shifted from 2.0% down again to 0.1%. The abrupt change in $^{15}X_N$ indeed confirmed the absence of label uniformity.

The results of the [^{15}N]nitrate-labelling experiments at $pO_2^R \geq 0.1\%$ prompted us to set up auxostat cultures permanently running at 2.0, 4.0, and 10% (vol/vol) oxygen. Indeed, our expectations of enforced rates of combined nitrification and denitrification under these aeration regimes were matched by the steady state N balances of the respective cultures: N losses significantly increased at higher pO_2^R, whereas free ammonium pools became almost depleted concurrently with higher concentrations of nitrate plus nitrite in the reactor fluid (Table 2).

Studies of the nature of the observed nitrifying activity involved different approaches. Because cooxidation of ammonia by methanotrophic species could be relevant in the auxostat culture at pO_2^R = 0.1% (i.e., because of significant CH_4 production [37]), $^{15}N_2$ emission from [^{15}N]ammonium was examined with 40% (vol/vol) CH_4 maintained in reservoir A (equivalent to ≈450 μM in the reactor fluid) and no supply of fresh

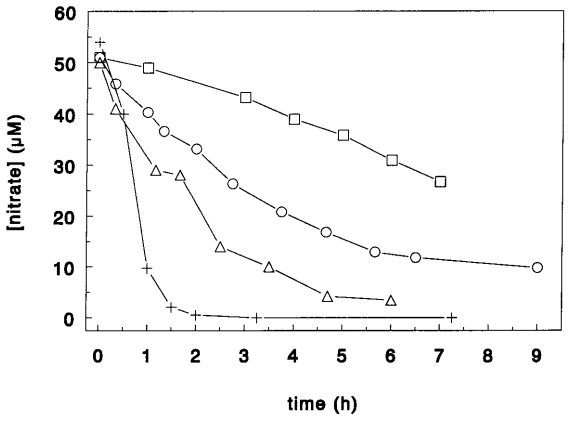

FIG. 2. Effect of temporal shifts in the oxygen partial pressure of the reactor slurry on the consumption rate of supplemented [^{15}N]nitrate in an auxostat running at a steady-state pO_2^R of 0.1% (vol/vol). Imposed pO_2^R (%, vol/vol); 0 (+), 0.5 (○), and 2.0 (□); control, 0.1 (△).

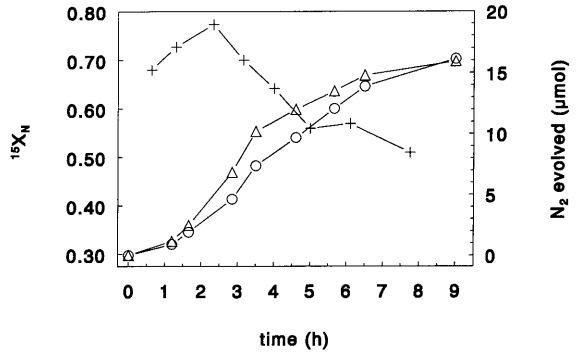

FIG. 3. Course of evolving $^{29}N_2$ (○) and $^{30}N_2$ (△) and of the apparent $^{15}X_N$ (+) after spiking [^{15}N]nitrate at pO_2^R = 0.5% (see Fig. 2 for the course of nitrate consumption at pO_2^R = 0.5%). $^{15}X_N$ was calculated from evolved $^{29}N_{2\ tx+1} - {}^{29}N_{2\ tx}$ and $^{30}N_{2\ tx+1} - {}^{30}N_{2\ tx}$.

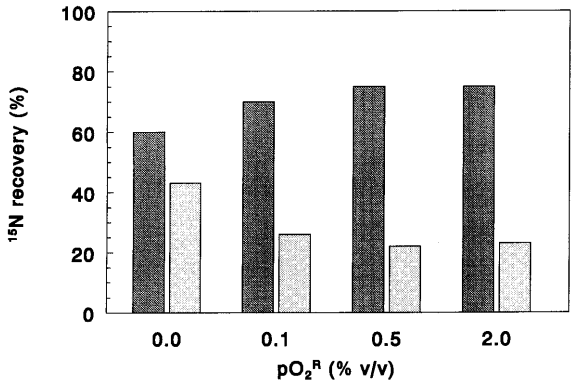

FIG. 4. Recovery of ^{15}N label from the free ammonium pool (light bar) and the headspace (dark bar) of an auxostat running at pO_2^R = 0.1% after consumption of spiked [^{15}N]nitrate at different imposed pO_2^R as shown in Fig. 2.

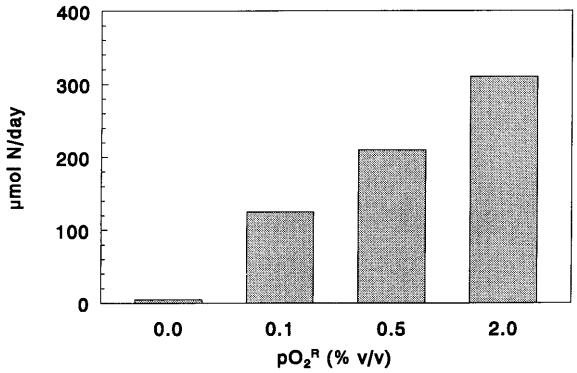

FIG. 5. Rate of nitrification as determined by [^{15}N]nitrate labelling at different pO_2^R imposed on the auxostat culture running at a steady-state pO_2^R of 0.1% (see the text for further details).

manure-straw. Although CH_4 was consumed at a rate of approximately 2 mmol · day^{-1}, no increase in the N_2 production was observed after 5 days of incubation, irrespective whether the supplemented CH_4 was omitted again or available at a reduced pressure of 4% (vol/vol). In fact, the production of N_2 was strongly reduced but was restored to its original value within a couple of days when the supply to the reactor had been resumed (data not shown).

Coupled nitrification-denitrification was also studied in the presence of inhibitors of autotrophic nitrification. To this end, nitrite and nitrate formation and $^{15}N_2$ evolution were monitored in [^{15}N]ammonium-spiked subcultures prepared from the auxostat at pO_2^R = 0.1%. None of the treatments with either acetylene (10 μM), dicyandiamide (90 μM), or chlorate (10 mM) was observed to exert an inhibitory effect on $^{15}N_2$ emission, although the percent N elimination (approximately 2%) in both control and treated cultures was low compared to auxostat performance. Some of the subcultures showed transient accumulations of nitrite and nitrate. However, the concentrations observed never exceeded 40 to 60 μM. Also, the enrichment cultures for autotrophic nitrifiers never accumulated substantial amounts of nitrite or nitrate (nitrite plus nitrate, <20 μM at t = 37 days). N elimination in these cultures was always less than 0.4%.

^{13}C labelling. A more rigorous test for the autotrophic or heterotrophic nature of the nitrifying population(s) would be the occurrence and the extent of CO_2 incorporation. Therefore, by a continuous labelling with [^{13}C]carbonate, the ^{13}C content of the CO_2-HCO_3^- system of the auxostat was raised by 0.2 atom%, corresponding to an increase in δ^{13}C of approximately +180‰. Because headspace gases are recirculated in the closed system of the auxostat, the isotopic composition of CO_2-HCO_3^- should be close to the δ^{13}C of the manure-straw substrate, i.e., in the range of −25.5 to −27‰ as determined by CF-IRMS analyses on the organic fraction of chicken manure and wheat straw, respectively. Hence, the δ^{13}C of microbes deriving their cell carbon largely from CO_2 was expected to be shifted toward a maximal value of +150‰, whereas the heterotrophic organisms, incorporating primarily manure-straw-derived carbon, should still reflect the isotopic composition of the carbon source (8). As shown in Table 3, the δ^{13}C of the microbial protein fraction increased 13‰ at pO_2^R = 0.1%. Although some FAMEs (e.g., peaks 11, 13, 14, 21, and 28) did take up ^{13}C label, the overall δ^{13}C of the lipid-derived fraction (weighted average) increased only approximately 1.5‰ from −26.84 to −25.19‰. Neither the δ^{13}C of the protein fraction nor the overall δ^{13}C of the FAME changed substantially upon a shift of pO_2^R from 0.1 to 2.0%, although FAME peaks 11, 13, 14, and 21 did show some further enrichment in ^{13}C. [^{13}C]carbonate labelling was repeated for the auxostat permanently running at pO_2^R = 4.0% after 6 months of operation. δ^{13}C enrichments of FAMEs exceeding 13‰ were not recorded (Table 4). Again, the monounsaturated fatty acids $C_{16:1\omega7c}$ (peak 14) and $C_{16:1\omega8}$ (peak 13) [^{13}C]carbonate label, and their relative contribution to the total FAME composition increased substantially. The cyclopropane fatty acids cy17:0 and cy19:0 could no longer be detected.

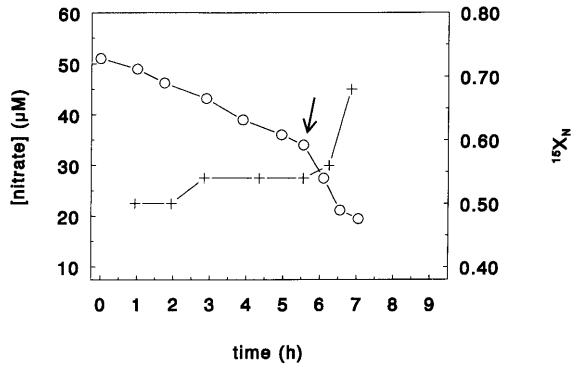

FIG. 6. Course of nitrate consumption (○) and the apparent $^{15}X_N$ (+) during a shift up (pO_2^R, 0.1 to 2.0%) and shift down (2.0 to 0.1%; indicated by the arrow) in the auxostat running at pO_2^R = 0.1%. At 5 h before addition of [^{15}N]nitrate (at t = 0 h), pO_2^R was raised from 0.1 to 2.0%.

TABLE 2. N budgets, nitrification rates, and steady-state concentrations of inorganic N species in the reactor fluid of auxostats running at different pO_2^R values

Parameter	Value in auxostat with pO_2^R of (%):			
	0.1	2.0	4.0	10
Duration of experiment	3 yr	1 yr	10 mo	6 wk
Influent input (ml · day^{-1})	450	500	650	500
$N_{Kjeld.}$ input (mmol · day^{-1})	2.7	3.0	3.9	3.0
$N_{Kjeld.}$ output (mmol · day^{-1})	2.1	1.9	2.5	1.6
[NH_4^+] in reactor fluid (mM)	2.5–3.0	<0.1	<0.05	<0.05
[NO_2^-] in reactor fluid (mM)	<0.01	0.2–0.3	0.2–0.5	0.2–0.3
[NO_3^-] in reactor fluid (mM)	<0.01	0.1–0.3	0.1–0.3	0.2–0.3
Rate of N loss (mmol · day^{-1})	0.6	1.1	1.4	1.4
Nitrification rate				
mmol of N · day^{-1} [a]	0.76	1.35	1.65	1.53
nmol of N · min^{-1} · mg of protein^{-1} [b]	7.0	12.4	15.1	14.0

[a] Calculated from the percent nitrate ammonification at each pO_2^R as determined by [^{15}N]nitrate labelling.

[b] Converted from the nitrification rate per millimole of N per day by using a reactor biomass of 0.06 mg of protein per milliliter, assuming an average cell number in the auxostats of 7×10^8 ml^{-1} (see Table 1) (6×10^9 cells = 1 mg [dry weight] [≈ 0.5 mg of protein]).

DISCUSSION

Our study showed that substantial N loss from a model composting system was not due to the volatilization of ammonia but was the result of combined nitrification and denitrification. Thermophilic methanotrophs were probably responsible for the observed nonautotrophic ammonia oxidation. To our knowledge, this is the first time that nitrification has been

TABLE 3. Carbon isotopic composition ($\delta^{13}C$) of saponifiable phospholipid FAs and cell protein in an auxostat running at pO_2^R = 0.1% upon [^{13}C]carbonate labelling

Peak	FA	pO_2^R = 0.1%				pO_2^R = 2.0%[a]	
		Relative %[b]	$\delta^{13}C$ (‰) at following ^{13}C atom% of CO_2-HCO_3^-[c]:			$\delta^{13}C$ (‰) at ^{13}C atom% of CO_2-HCO_3^- of 1.28[c]	Relative %[b]
			1.08	1.18	1.28		
3	12:0	1.5	−27.97	−29.97	−26.40	−26.31	1.2
5	i14:0	0.5	−26.33	−25.60	−24.46	−22.62	0.4
7	14:0	3.7	−29.14	−30.35	−28.66	−26.51	3.7
8	i15:0	4.0	−25.44	−23.69	−22.69	−21.06	5.4
9	a15:0	3.8	−24.79	−23.97	−22.74	−23.46	3.1
11	i16:0	2.4	−28.53	−25.40	−25.35	−22.66	3.1
13	16:1ω8	0.8	−28.60	−25.35	−22.24	−18.77	0.8
14	16:1ω7c	1.3	−31.67	−28.48	−26.15	−22.00	1.5
15	16:0	20.6	−27.25	−27.99	−25.79	−25.20	20.9
17	i17:0	4.5	−26.38	−26.36	−24.58	−25.00	4.4
21	cy17:0	0.7	−23.15	−20.77	−18.32	−14.60	0.4
25	18:2ω6,9	15.6	−24.70	−24.66	−24.08	−23.36	14.9
26	18:1ω9c	21.2	−26.75	−26.44	−26.55	−26.20	21.1
27	18:0	6.2	−26.00	−26.75	−24.41	−23.87	6.0
28	cy19:0	2.3	−26.23	−20.46	−14.36	−15.35	3.5
30	20:0	0.4	−30.59	−30.22	−28.21	−29.14	0.3
32	20:2	2.5	−27.59	−25.66	−26.12	−25.60	2.2
	Total FA		−26.84	−26.01	−25.19	−24.31	
	Cell protein[d]		≈−27.0	ND[e]	≈−14.0	≈−13.0	

[a] FAME analysis after 2.5 culture turnovers following the pO_2^R shift from 0.1 to 2.0%.
[b] Percentage of total FAME carbon present.
[c] ^{13}C atom% of CO_2-HCO_3^- at the start of the [^{13}C]carbonate labelling estimated from $\delta^{13}C$ of total FAs and cell protein, assuming that CO_2 from respiration reflects the isotopic composition of the substrates used for growth.
[d] HCl-precipitable protein fraction of total reactor contents.
[e] ND, not determined.

demonstrated to exist (as a quantitative important process) at temperatures exceeding 40°C.

The presence of autotrophic nitrifiers in the auxostat active at 53°C, was not expected in view of the numerous unsuccessful attempts to enrich or isolate such organisms from thermophilic environments by others (1, 11, 16). Indeed, in our hands, the enrichment for thermophilic autotrophic nitrifiers also turned out negative; we did not observe accumulation of nitrite or nitrate, and we could not detect evolution of $^{15}N_2$ (i.e., no false negatives by aerobic denitrification). Ammonia oxidation in the auxostats, therefore, is most likely to be of heterotrophic origin. This conclusion is corroborated by the observed decreasing nitrifying activity when the supply of fresh slurry to the reactor is stopped (and its subsequent restoration after resumption of the slurry supply), since nitrification by mesophilic heterotrophs is known to require the presence of an external carbon and energy source in many cases (40).

TABLE 4. Carbon isotopic composition ($\delta^{13}C$) of saponifiable phospholipid FAs in auxostat running at pO_2^R = 4.0% for 6 months upon [^{13}C]carbonate labelling

FA	Relative %[a]	$\delta^{13}C$ (‰) at following ^{13}C atom% of CO_2-HCO_3^-[b]:		
		1.08	1.18	1.28
14:0	3.6	−23.69	−24.32	−23.67
i15:0	9.0	−25.34	−23.60	−24.27
a15:0	3.6	−25.19	−23.94	−22.72
i16:0	4.4	−24.73	−22.93	−24.91
16:1ω8	7.3	−23.28	−18.50	−10.10
16:1ω7c	5.8	−22.25	−18.48	−12.72
16:0	32.0	−26.13	−26.36	−24.25
a17:0	2.7	−26.38	−26.36	−24.58
18:2ω6,9	5.4	−32.38	−33.85	−30.02
18:1ω9c	9.0	−29.96	−28.96	−27.48
18:0	5.9	−28.94	−27.63	−25.72
20:0	2.4	−30.59	−30.22	−28.21
20:2	2.6	−27.59	−25.66	−26.12
Total FA		−26.84	−26.01	−25.19

[a] Percentage of total FAME carbon present.
[b] ^{13}C atom% of CO_2-HCO_3^- at the start of the [^{13}C]carbonate labelling estimated from $\delta^{13}C$ of total FAs, assuming that CO_2 from respiration reflects the isotopic composition of the substrates used for growth.

Many heterotrophic nitrifying bacteria also simultaneously denitrify (41). Both nitrification and denitrification were reported to increase with decreasing dissolved oxygen concentration for *Thiosphaera pantotropha*, without accumulation of nitrite or nitrate in the culture fluid (41). The occurrence in the auxostats of such an intimate coupling of both activities, mediated by a single population, is not supported by our data. The time needed (≈2 h) for reaching an apparent isotopic equilibrium in the [^{15}N]nitrate-labelling experiments (Fig. 3) indicates that the sites for denitrification are spatially separated from the bulk fluid. Hence, this activity resides in the interior of aggregates or organic solids, probably devoid of oxygen at the imposed low pO_2 of the reactor fluid. The presence of poorly equilibrating nitrate pools in the auxostat was clearly demonstrated by the sudden increase in apparent $^{15}X_N$, following a shift of pO_2^R from 2.0 to 0.1% (Fig. 6). From this, we conclude that the nitrifiers are localized between the bulk fluid and the denitrifying population(s), i.e., in the outer (oxic) layer of aggregates and solids. If they are located elsewhere, no rational can be given why the [^{14}N]nitrate from nitrification distributes unevenly with ^{15}N-enriched nitrate of the reactor fluid prior to

its uptake by denitrifiers. Preferential utilization of the [^{14}N]nitrate as a result of the proximity of nitrifying and denitrifying populations in aggregates also explains the strong underestimation of the nitrifying activity determined by means of [^{15}N]nitrate labelling.

Methanotrophs have also been implicated in the occurrence of nonautotrophic nitrification (5). A CH_4-dependent ammonia oxidation, as observed by others (14, 29, 42), would explain the declining nitrification activity in the auxostat in the absence of medium supply (ceasing CH_4 formation) and its substantial reduction (±10-fold) in the batch subcultures (O_2-inhibited methanogenesis). Raising the ambient CH_4 concentration in the auxostat from 0.3–0.7 μM to 45 or 450 μM did not enhance ammonia oxidation but abolished it instead. Inhibition of methanotrophic nitrification at comparable levels of CH_4 has also been reported (36, 43). A clue to the identity of the population(s) responsible for the observed nitrification was provided by [^{13}C]carbonate labelling of the auxostat. CO_2 fixation was evident from ^{13}C enrichment of the protein fraction by approximately 13‰. Since the $\delta^{13}C$ of the CO_2-HCO_3^- was raised by 180‰, this fixation amounts to $13/180 \times 100\% \approx 7\%$ of culture biomass carbon, a value in the range expected for anapleurotic CO_2 fixation. Accordingly, since anapleurotically fixed CO_2 is not funneled toward straight-chain and cyclopropane FAs, enrichment of the total FA fraction is much less, only 1.6 to 2.5‰. FAs that deviate from this level of enrichment, such as peaks 13, 14, 21, and 28 (Table 4), must have a different biosynthetic background. The genus *Methylococcus* encompasses moderately thermophilic species such as *Methylococcus capsulatus* and *M. thermophilus*, with optima for growth in the range of 40 to 50°C. In contrast to other methanotroph genera, members belonging to this genus contain a partially functional Benson-Calvin cycle which allows the incorporation of carbon dioxide in the presence of CH_4 (i.e., type X group). According to lipid profile analysis of the genus *Methylococcus* by Bowman et al. (7) the best fit based on the ^{13}C-enriched FAMEs mentioned above would be for the so-called phenon 11 group. Apart from the methanotroph-related $C_{16:1\omega7c}$, cy17:0 and cy19:0 may also occur as (major) FA in the three *Methylococcus* phena discerned by Bowman et al. (7). $C_{16:1\omega8}$, however, is a unique FA confined to phenon 11.

Raising pO_2^R from 0.1 to 2.0% brought about an increase of the nitrification rate and a significant further enrichment in ^{13}C for the FA $C_{16:1\omega7c}$, $C_{16:1\omega8}$ and cy17:0, suggesting a metabolic interlinkage. Since K_m values for oxygen of the methane monooxygenase range from 10 to 20 μM (19, 22), the (nitrifying) activity of the methanotrophs at $pO_2^R = 0.1\%$ probably was constrained by the ambient low oxygen concentration (≈0.8 μM). Relief of the limitation enhanced the oxidation of ammonia and may well have resulted in a metabolic shift in the assimilation of carbon from CO_2, giving rise to the cellular ^{13}C increment. At $pO_2^R = 4.0\%$ (vol/vol), $C_{16:1\omega7c}$ and $C_{16:1\omega8}$ again were enriched upon $^{13}CO_2$ labelling (Table 4). The increase in relative abundance of these two FAs at the cost of cy17:0 and cy19:0, which completely disappeared, is in agreement with the reported oxygen dependence of the membrane FA composition of *M. capsulatus* (21). The sixfold increase in the abundance of $C_{16:1\omega7c}$ and $C_{16:1\omega8}$ strongly suggests that the population of methanotrophs had proliferated at $pO_2^R = 4.0\%$ (vol/vol).

At first sight, it may seem surprising that methanotrophs could maintain themselves in auxostats running at $pO_2 \geq 2.0\%$, since CH_4 formation was shown to no longer occur under these conditions. However, methanotrophs belonging to the genus *Methylococcus*, and especially those of phena 10 and 11, are capable of utilizing carbon sources other than CH_4, i.e., formate, formamide, and methylamines (7). Methylated amines occur as degradation products of carnitine and choline derivatives under anoxic conditions (15), and trimethylamine has been implicated as an odoriferous compound formed during composting (30). In addition, we would like to recall the findings on the possible functioning of a lithotrophic pathway of energy production in *Methylococcus* species by Malashenko et al. in the late 1970s (26). The presence in these species of a hydroxylamine oxidoreductase, sharing a number of properties with the corresponding enzyme in ammonia oxidizers, was demonstrated (9, 47). Despite the apparent constitutive nature of the hydroxylamine oxidoreductase in the group of methylococci and the substrate specificity of the enzyme (47), it has remained unclear whether NH_2OH oxidation can contribute significantly to the energy budget of methanotrophs which possess this activity. It is tempting to speculate that under the conditions provided by the auxostats, this ability has evolved or has been optimized as a result of the prolonged carbon and energy limitation imposed on the methanotrophs initially present. In particular, the pO_2^R shift from 0.1 to 2.0% may have brought about additional pressure on the methanotrophic populations because of the cessation of methane formation at this oxygen level in the reactor. In fact, the nitrification rates at $pO_2^R \geq 2.0\%$ (Table 2) are in between the rates reported for chemostat cultures of the autotrophic *Nitrosomonas europaea* and the heterotrophic nitrifier *T. pantotropha* at comparable growth rates: 675 and 4.2 nmol of $N \cdot min^{-1} \cdot$ mg of protein^{-1}, respectively (data from references 3 and 25). Obviously, since the auxostat consists of a mixed culture, the cell-specific activity of the nitrifying population has to be substantially higher than 15.1 nmol of $N \cdot min^{-1} \cdot$ mg of protein^{-1} and, indeed, could be approaching the rates reported for mesophilic autotrophic nitrifiers.

The affinity of the methane monooxygenase enzyme for ammonia has led to speculations regarding the contribution of methanotrophs to nitrification in the environment (24). Evidence for such an involvement, however, is still limited (29, 43). The picture that emerges from recent work on the mutuality of methane and ammonium metabolism in natural habitats (13, 23, 43) suggests that the occurrence and extent of a methanotrophic nitrification will be highly dependent on site-specific conditions, especially the local methane and ammonium concentrations. Our results strongly support the notion that, at least under thermophilic conditions, methanotrophs indeed are capable of a quantitatively important oxidation of ammonia. This extensive oxidation of ammonia even occurs in the absence of methane, suggesting that elements of a lithotrophic metabolism are involved. Detailed knowledge of carbon mineralization and nitrogen transformations under suboxic conditions, including the description of the thermophilic microbial consortia involved, may eventually permit an optimization of the process parameters enabling a control over the release of ammonia. It would be especially worthwhile to explore the introduction at the start of a compost run of methanotrophic consortia, as described in this paper, to prime the process of methanotrophic nitrification and to provide sufficient oxidative capacity at the onset of the thermophilic stage with its associated enhanced ammonia volatilization.

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