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Methane Oxidation by *Nitrosococcus oceanus* and *Nitrosomonas europaea*†

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Chemolithotrophic ammonium-oxidizing and nitrite-oxidizing bacteria including *Nitrosomonas europaea, Nitrosococcus oceanus, Nitrobacter* sp., *Nitiospina gracilis*, and *Nitrococcus mobilis* were examined as to their ability to oxidize methane in the absence of ammonium or nitrite. All ammonium oxidizers tested had the ability to oxidize significant amounts of methane to CO_2 and incorporate various amounts into cellular components. None of the nitrite-oxidizing bacteria were capable of methane oxidation. The methane-oxidizing capabilities of *Nitrosococcus oceanus* and *Nitrosomonas europaea* were examined with respect to ammonium and methane concentrations, nitrogen source, and pH. The addition of ammonium stimulated both CO_2 production and cellular incorporation of methane-carbon by both organisms. Less than 0.1 mM CH_4 in solution inhibited the oxidation of ammonium by *Nitrosococcus oceanus* by 87%. Methane concentrations up to 1.0 mM had no inhibitory effects on ammonium oxidation by *Nitrosomonas europaea*. In the absence of NH_4-N, *Nitrosococcus oceanus* achieved a maximum methane oxidation rate of 2.20×10^{-2} μmol of CH_4 h^{-1} mg (dry weight) of cells^{-1}, which remained constant as the methane concentration was increased. In the presence of NH_4-N (10 ppm [10 μg/ml]), its maximum rate was 26.4×10^{-2} μmol of CH_4 h^{-1} mg (dry weight) of cells^{-1} at a methane concentration of 1.19×10^{-2} mM. Increasing the methane concentration above this level decreased CO_2 production, whereas cellular incorporation of methane-carbon continued to increase. *Nitrosomonas europaea* showed a linear response throughout the test range, with an activity of 196.0×10^{-2} μmol of CH_4 h^{-1} mg (dry weight) of cells $^{-1}$ at a methane concentration of 1.38×10^{-1} mM. Both nitrite and nitrate stimulated the oxidation of methane. The pH range was similar to that for ammonium oxidation, but the points of maximum activity were at lower values for the oxidation of methane.

Ammonium oxidation by chemolithotrophic ammonium oxidizers and methane oxidation by methane-oxidizing bacteria have been shown to be quite similar (5, 13, 14). Many methane-oxidizing bacteria will oxidize ammonium to nitrite (10, 13). This ability is linked to the lack of specificity of the methane oxygenase system (5, 10). The oxidations of ammonium and methane both start with the hydroxylation of the substrates (14). Despite these similarities, prior research using oxygen uptake measurements has indicated that *Nitrosomonas europaea* is unable to oxidize methane (4, 11). However, with [^{14}C]methane, we found that ammonium oxidizers including *Nitrosomonas europaea* and *Nitrosococcus oceanus* are able to oxidize significant amounts of methane to $^{14}CO_2$ and incorporate some of the carbon from methane into cellular components.

Ammonium oxidizers are considered to be metabolically limited to a very few substrates (3, 15), and their ability to oxidize methane poses interesting questions as to the interactions between the oxidation of ammonium and methane.

This paper addresses the ability of ammonium oxidizers to incorporate methane into cellular components as well as respire the methane to carbon dioxide in the presence and absence of ammonium and other nitrogen sources.

MATERIALS AND METHODS

Cultures and inoculum. Cultures of *Nitrosococcus oceanus, Nitrosomonas marinas* sp. strain C-15, *Nitrococcus mobilis, Nitrospina gracilis, Nitrobacter* sp. strain Nb297 (provided by S. W. Watson), *Nitrosomonas europaea* (provided by E. L. Schmidt), and several of our isolates representing marine, estuarine, freshwater, and soil nitrifiers were used in this study. The ammonium oxidizers were grown and assayed for

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purity, using the medium and continuous-flow methods described by Jones and Hood (9). Nitrite oxidizers were cultured in an identical manner, using the medium described by Watson and Waterbury (12) with seawater being replaced by Instant Ocean synthetic sea salts (Aquarium Systems, Inc.) in distilled water to give a salinity of 32‰. A standard inoculum was prepared by filtering cells onto a membrane filter (type HA, 0.45 μm; Millipore Corp.). The cells were washed twice with sterile NH_4- or NO_2-free medium, pH 7.8, and resuspended to an optical density of 0.2 at 550 nm (Bausch & Lomb Spectronic 20 spectrophotometer). Portions (1 ml) of these suspensions were used as the inoculum to test for methane oxidation by the various nitrifiers.

The medium used for preparing these suspensions and for methane oxidation determinations had the following composition: $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; K_2HPO_4, 0.114 g; Fe-EDTA (77 mg of $FeSO_4 \cdot 7H_2O$ + 103 mg of Na EDTA in 50 ml of distilled water), 1.0 ml; $Na_2MoO_4 \cdot 2H_2O$, 1.0 μg; $COCl_2 \cdot 6H_2O$, 2.0 μg; $ZnSO_4 \cdot 7H_2O$, 100 μg; K_2CO_3, 0.1 g; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Sigma Chemical Co.), 2.4 g; distilled water, 1.0 liter. The pH of the medium was adjusted to 7.8 with 5 N NaOH. The salinity of the medium was adjusted with Instant Ocean synthetic sea salts to 10‰ for *Nitrosomonas* sp. strain 1S10, 18‰ for *Nitrosomonas marinus* sp. strain C-15, and 32‰ for the remaining marine isolates.

Suspensions of *Nitrosococcus oceanus* and *Nitrosomonas europaea* used for the evaluation of the interactions of methane and ammonium were subjected to cell counts, using acridine orange fluorescent microscopy (8) and dry weight determinations. For dry weights, 5.0 ml of culture was filtered onto a predried preweighed Millipore membrane filter (type HA, 0.45 μm) and dried at 80°C until a constant value was obtained.

The mean density of *Nitrosococcus oceanus* suspensions prepared in the described manner was 1.1×10^8 cells ml^{-1}, with an average dry weight of 36 μg ml^{-1}. The values for *Nitrosomonas europaea* suspensions were 3.2×10^8 cells ml^{-1} and 26 μg ml^{-1}. One-milliliter portions of these suspensions were used as the standard inoculum.

Methane oxidation determinations. The method used for $^{14}CH_4$ preparation and methane oxidation rate determinations was similar to those described by Griffiths et al. (6). A 1-ml amount of standard inoculum was inoculated into 60-ml serum bottles containing 25 ml of NH_4-free medium buffered with 0.2 M HEPES, pH 7.8. The bottles were then sealed with serum stoppers, and 1.0 ml (unless otherwise noted) of $^{14}CH_4$ (1.0 μCi ml^{-1}; specific activity, 59 mCi $mmol^{-1}$; Amersham Corp.) in nitrogen was added to the head space. The final concentration of $^{14}CH_4$ in solution ranged from 13.8 nM for the *Nitrosomonas europaea* (freshwater) cultures to 11.9 nM for the *Nitrosococcus oceanus* (marine) cultures (16). All bottles were prepared in triplicate and all experimental sets were duplicated. Controls without cells and with acid-killed cells were run with each experiment. The bottles were incubated for 48 h at 25°C at 100 rpm on a rotary shaker. After incubation, $^{14}CO_2$ production and ^{14}C incorporated into cellular material were measured, and methane oxidation rates were determined from these data. Confidence intervals were calculated for each data point. The variation among replicates did not exceed the 95% confidence interval for the population mean.

Once the ability of ammonium oxidizers to oxidize methane was determined, further detailed examinations of *Nitrosococcus oceanus* and *Nitrosomonas europaea* were made with respect to ammonium and methane concentrations, nitrogen source, and pH.

To test the effects of ammonium, a series of bottles containing 0, 1, 10, 50, and 100 ppm (μg/ml) of NH_4-N as $(NH_4)_2SO_4$ was prepared and incubated as previously described.

Bottles containing various nitrogen sources were prepared to examine their effects on methane oxidation. The following additions were made: 10 ppm of NH_4-N, 10 and 40 ppm of NO_2-N, 10 and 40 ppm of NO_3-N, and 10 ppm of yeast extract (Difco Laboratories). Controls which contained no additional nitrogen source were also run.

The effects of methane concentration on methane oxidation were examined. A series of bottles containing 0.0001, 0.001, 0.01, 0.1, 0.5, 1.0, 5.0, and 10% methane head spaces was prepared. The concentrations of 0.0001 and 0.001% CH_4 were prepared by injecting 0.1 and 1.0 ml of the $^{14}CH_4$-N_2 mixture. The higher methane concentrations were prepared by injecting 1.0 ml of $^{14}CH_4$-N_2 and 3.4, 38, 190, 1,900, and 3,800 μl of unlabeled methane into the head space to make up the balance. The addition of unlabeled methane resulted in a serial dilution of specific activity. It was assumed that there would be no significant isotopic fractionation by the microorganisms, and therefore only the dilution effects were taken into account when calculating activity. Methane concentrations ranged from 1.19×10^{-6} to 1.19×10^{-1} mM for *Nitrosococcus oceanus* and 1.38×10^{-6} to 1.38×10^{-1} mM for *Nitrosomonas europaea*. Methane series were prepared both with and without 10 ppm of NH_4-N.

A pH series was prepared by adjusting the medium to various pH values between 4.0 and 11.0, using either 1.0 N HCl or 1.0 N NaOH. Series were prepared both with and without the addition of 10 ppm of NH_4-N. Bottles containing ammonium were assayed for nitrite production, using the spectrophotometric method of Bendschneider and Robinson (2), to determine ammonium oxidation.

The effects of dissolved carbonate concentration on cellular incorporation of C from $^{14}CH_4$ were determined by preparing a series of bottles that contained 0, 10, 50, 200, and 500 ppm of dissolved carbonate as K_2CO_3. The dissolved carbonates were first removed from the medium by acidification to pH 2.0 with HCl and then boiling for 10 min. The solution was then allowed to return to room temperature and adjusted to pH 7.0 with 1.0 N NaOH. Carbonate additions were made and the final pH was adjusted to 7.8 with 1.0 N NaOH or HCl. The bottles were then sealed immediately, and the $^{14}CH_4$, 10 ppm of NH_4-N, and cells were added.

Carbonate uptake. Bottles were prepared as usual, and then $Na_2H^{14}CO_3$ was added (specific activity, 4.5 μCi $mmol^{-1}$; Amersham Corp.) to give a final concentration of 0.01 μCi ml^{-1}. The bottles were sealed and 0, 0.1, 1.0, or 10% CH_4 was added to the head space. One set of bottles was given an addition of 1.0 ppm of NH_4-N. The bottles were then incubated as usual and analyzed for ^{14}C incorporation.

Methane effects on ammonium oxidation. A 1-ml amount of standard inoculum was inoculated into 60-ml serum bottles which were then filled with 1.0 ppm of NH_4-N–0.02 M HEPES-buffered (pH 7.8) medium and capped in such a manner as to exclude any air bubbles. A 1-ml head space was then created by injecting 1.0 ml of various CH_4-N_2 (Airco, grade 4, ultrapure gas) mixtures and removal of 1.0 ml of medium. The methane concentrations in the injected gases were 0, 1, 10, 50, and 100%, giving a concentration range of 0.0 to 1.38 mM dissolved methane for *Nitrosomonas europaea* and 0.0 to 1.119 mM for *Nitrosococcus oceanus* (16). These bottles were then incubated for 12 h at 25°C at 100 rpm on a rotary shaker. The bottles were then assayed for nitrite. All bottles were prepared in triplicate, and each experimental set was duplicated.

RESULTS

The rates of methane oxidation by each organism are shown in Table 1. All of the ammonium oxidizers examined were able to oxidize methane to CO_2 and incorporate it into cellular components, in the absence of ammonium. The nitrite oxidizers examined were unable to oxidize methane. The rate of methane oxidization under these conditions varied significantly between species, with *Nitrosococcus oceanus* having the highest rate and *Nitrosomonas* sp. strain 2SO the lowest. The ratio of methane-carbon incorporated into cellular material to that of CO_2 produced from methane also varied significantly. With the exception of *Nitrosomonas* sp. strain 3S30, an Oregon coast isolate, all of the marine isolates had a lower incorporation rate than the freshwater, soil, and estuarine isolates.

The presence of ammonium stimulated the production of $^{14}CO_2$ and ^{14}C-cellular C by both *Nitrosococcus oceanus* and *Nitrosomonas europaea* (Fig. 1). *Nitrosomonas europaea* was stimulated to a greater extent, but both organisms showed the same trends. A 10-ppm portion of NH_4-N caused the greatest increases in methane oxidation, with further increases in ammonium concentration causing a decrease in activity.

In addition to being stimulated by ammonium, nitrite, nitrate, and yeast extract also stimulated methane oxidation (Table 2). Increasing the concentrations of NO_2^- and NO_3^- from 10 to 40 ppm did not cause any further increase in methane oxidation.

The response of *Nitrosococcus oceanus* to methane concentration both with and without 10 ppm of NH_4-N is shown in Fig. 2. In the absence of ammonium, both $^{14}CO_2$ production and ^{14}C-cellular incorporation from $^{14}CH_4$ showed a linear response to increasing methane concentrations up to 1.19×10^{-3} mM CH_4. At this point ^{14}C-cellular incorporation continued to increase, whereas $^{14}CO_2$ production remained essentially constant. In the presence of 10 ppm of ammonium, ^{14}C-cellular incorporation showed a similar linear trend, whereas $^{14}CO_2$ production showed linearity to 1.19×10^{-2} mM and then decreased with further increases in methane concentration. The response of *Nitrosomonas europaea* to methane concentration is shown in Fig. 3. In both the presence and absence of ammonium the response of *Nitrosomonas europaea* to methane concentration was linear. In the absence of ammonium at methane concentrations above 1.38×10^{-2} mM, the cellular incorporation of $^{14}CH_4$-C exceeded the amount of $^{14}CO_2$ produced.

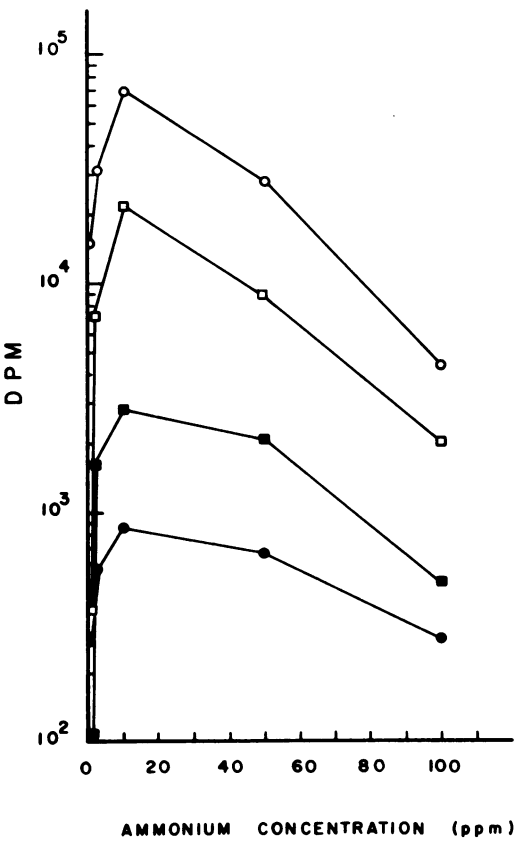

FIG. 1. Ammonium effects on methane oxidation. Activity is expressed as dpm of ^{14}C ml of standard inoculum^{-1} 24 h^{-1}. Symbols: (○) *Nitrosococcus oceanus*, $^{14}CO_2$ produced; (●) *N. oceanus*, ^{14}C-cellular material; (□) *Nitrosomonas europaea*, $^{14}CO_2$ produced; (■) *N. europaea*, ^{14}C-cellular material.

The ratios of methane incorporated into cellular material to that released as CO_2 are shown in Table 3. In the absence of ammonium the values for *Nitrosococcus oceanus* remained essentially constant until a methane concentration of 0.595 $\times 10^{-2}$ mM; at this point the ratio increased until 48.8% of the methane oxidized was incorporated

TABLE 1. Methane oxidation by nitrifiers in the absence of ammonium

Organism	Source	Methane oxidation rate (dpm)[a]		Ratio ^{14}C-cellular material/$^{14}CO_2$
		$^{14}CO_2$ produced	^{14}C-cells	
Nitrosococcus oceanus	Marine, North Atlantic	15,051	293	0.019
Nitrosomonas europaea	Soil	596	60	0.101
Nitrosomonas marinus sp. strain C-15	Marine, South Pacific	233	13	0.055
Nitrosomonas sp. strain 1S10	Estuarine, Florida coast	924	194	0.210
Nitrosomonas sp. strain 2S0	Freshwater, Louisiana marsh	179	19	0.106
Nitrosomonas sp. strain 6S30	Marine, Alaskan coast	1,285	27	0.021
Nitrosomonas sp. strain 9W0	Freshwater, Oregon marsh	782	85	0.109
Nitrosomonas sp. strain 3S30	Marine, Oregon coast	2,590	370	0.143
Nitrosomonas sp. strain 11W30	Marine, Oregon coast	2,983	51	0.017
Nitrobacter sp. strain Nb297	Marine	3	0	
Nitrospina gracilis	Marine, South Atlantic	0	1	
Nitrococcus mobilis	Marine, South Pacific	1	0	

[a] Activity is expressed as dpm of ^{14}C ml of standard inoculum^{-1} 24 h^{-1}.

into cellular material at 1.19×10^{-1} mM CH_4. In the presence of 10 ppm of NH_4-N, the ratio remained constant until a methane concentration of 0.595×10^{-1} nM, at which point it increased an order of magnitude and then increased further until 75.7% of the methane oxidized was incorporated into cellular carbon at 1.19×10^{-1} mM CH_4. *Nitrosomonas europaea* also incorporated more ^{14}C as the methane concentration increased. In the absence of ammonium in the presence of methane concentrations above 1.38×10^{-2} mM, more ^{14}C was incorporated than released as $^{14}CO_2$. With ammonium, the incorporation of methane carbon ranged from 20.7% at 1.38×10^{-5} mM to 31.6% at 1.38×10^{-1} mM.

Nitrosococcus oceanus in the absence of ammonium oxidized 2.20×10^{-2} μmol of CH_4 h^{-1} mg (dry weight) of cells^{-1} at 1.0% methane head space (1.19×10^{-2} mM CH_4 dissolved) and 26.4 $\times 10^{-2}$ μmol in the presence of 10 ppm of ammonium at the same methane concentration. *Nitrosomonas europaea* with a 10% methane head space (1.38×10^{-1} mM CH_4 dissolved) oxidized 11.1 μmol of CH_4 h^{-1} mg (dry weight) of cells^{-1} in the absence of ammonium and 196.0 $\times 10^{-2}$ μmol in the presence of ammonium.

The responses of methane oxidation by both organisms to pH were quite similar. *Nitrosococcus oceanus* had an optimum range for ammonium oxidation of pH 8.0, whereas methane oxidation occurred optimally at pH 7.0 (Fig. 4). *Nitrosomonas europaea* had an optimum for ammonium oxidation of pH 9.5, whereas methane oxidation occurred optimally at pH 7.5 (Fig. 5). Both organisms exhibited a broad range of high methane oxidation activity in the presence or absence of ammonium.

Increasing the carbonate concentration in solution caused a corresponding decrease in the amount of $^{14}CH_4$-C incorporated into cellular material in cultures containing ammonium (Table 4).

The uptake of $^{14}CO_3$ by either *Nitrosococcus oceanus* or *Nitrosomonas europaea* was not increased by the addition of methane at any concentration, in the absence of ammonium.

The effects of methane concentration on ammonium oxidation were quite different for the two organisms. The ability of *Nitrosococcus*

TABLE 2. Effects of ammonium, nitrite, nitrate, and yeast extract on methane oxidation by *Nitrosococcus oceanus* and *Nitrosomonas europaea*

Organism	Fraction	Methane oxidation rate (dpm)[a] with given addition						
		None	NH_4-N (10 ppm)	NO_2-N		No_3-N		Yeast extract (10 ppm)
				10 ppm	40 ppm	10 ppm	40 ppm	
Nitrosococcus oceanus	$^{14}CO_2$	15,558	76,020	39,416	38,973	34,776	32,337	61,395
	^{14}C-cells	240	575	43	ND[b]	379	ND	931
Nitrosomonas europaea	$^{14}CO_2$	674	26,783	1,618	1,792	1,159	1,170	3,513
	^{14}C-cells	31	3,383	320	ND	286	ND	543

[a] Activity is expressed as dpm of ^{14}C ml of standard inoculum^{-1} 24 h^{-1}.
[b] ND, Not determined.

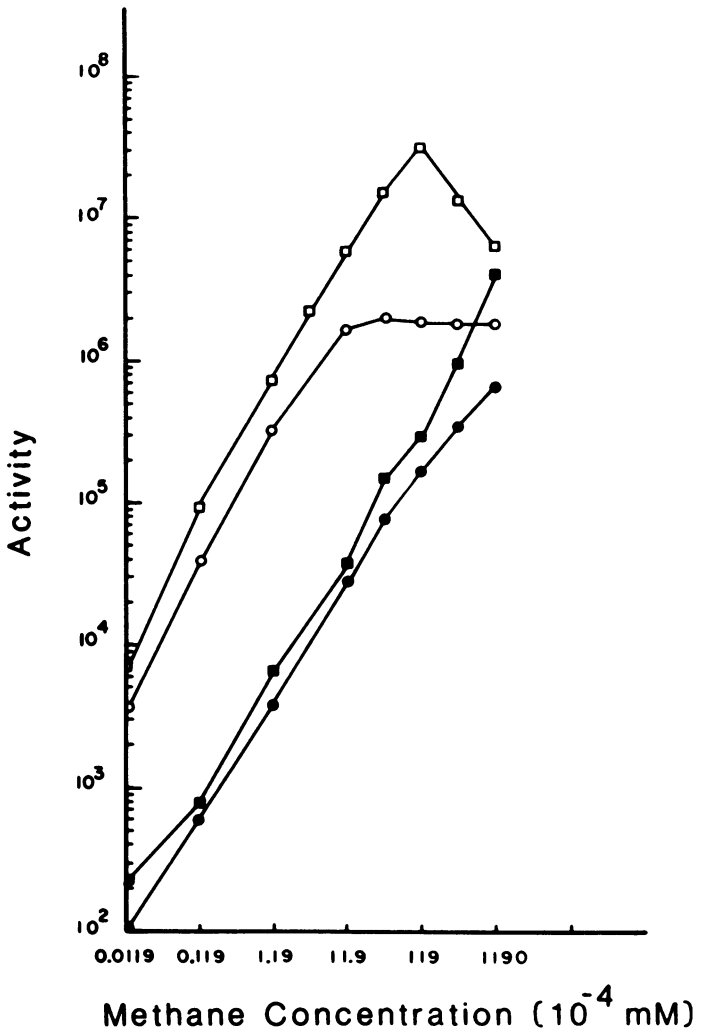

FIG. 2. Effects of methane concentration on rate of methane oxidation by *Nitrosococcus oceanus*. Activity is expressed as dpm of ^{14}C ml of standard inoculum^{-1} 24 h^{-1} multiplied by the dilution factor. Symbols: (○) $^{14}CO_2$ produced, 0.0 ppm of NH_4-N; (●) ^{14}C-cellular material, 0.0 ppm of NH_4-N; (□) $^{14}CO_2$ produced, 10 ppm of NH_4-N; (■) ^{14}C-cellular material, 10 ppm of NH_4-N.

oceanus to oxidize ammonium was inhibited by methane concentrations of <0.115 mM. Ammonium oxidation by *Nitrosomonas europaea* was not affected by any of the methane concentrations (Fig. 6).

DISCUSSION

The similarities between ammonium oxidizers and methane oxidizers have been noted by several other workers (5, 13, 14). Methane oxidizers have also been shown to oxidize ammonium to nitrite (10, 13), although the rate of this process is extremely slow. Work by Drozd (4) and Suzuki et al. (11), using the relatively insensitive method of oxygen uptake, were unable to detect any oxidation of methane by *Nitrosomonas europaea* and concluded that no methane oxidation took place. With a more sensitive method involving the use of $^{14}CH_4$, the results of this research show that ammonium oxidizers including *Nitrosomonas europaea* can oxidize methane. Whether this is due to the lack of specificity of the ammonium oxygenase system, which seems likely, or whether separate enzymes responsible for methane oxidation exist is not known. Prior work by Suzuki et al. (11) has indicated that oxidation of ammonium by *Nitrosomonas europaea* was inhibited by methane. We were unable to duplicate this with our strain

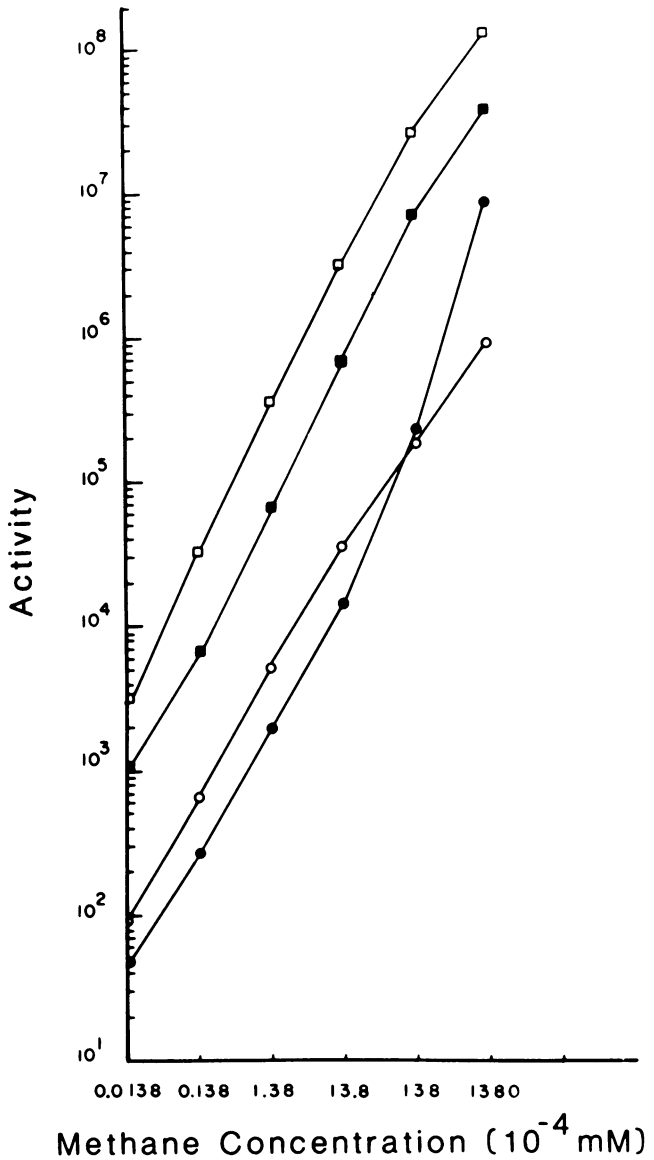

FIG. 3. Effects of methane concentration on rate of methane oxidation by *Nitrosomonas europaea*. Activity is expressed as dpm of ^{14}C ml of standard inoculum^{-1} 24 h^{-1} multiplied by the dilution factor. Symbols: (○) $^{14}CO_2$ produced, 0.0 ppm of NH_4-N; (●) ^{14}C-cellular material, 0.0 ppm of NH_4-N; (□) $^{14}CO_2$ produced, 10 ppm of NH_4-N; (■) ^{14}C-cellular material, 10 ppm of NH_4-N.

of *Nitrosomonas europaea*. *Nitrosococcus oceanus* did show an inhibitory response similar to that reported by Suzuki et al. (11). This indicates a marked difference between the two organisms. The stimulation of methane oxidation by low concentrations of ammonium seems to indicate that the interactions between ammonium and methane are more than simple competitive inhibition; in fact, no inhibitory response was observed for *Nitrosomonas europaea*. Ammonium concentrations above 10 ppm of NH_4-N decreased the methane oxidation activity for both organisms, indicating a sort of one-sided "competitive inhibition." These relationships may be due in part to the relative insolubility of methane. Part of the increased activity caused by the addition of low levels of ammonium may be due to an activation of the enzyme system; the other could be due to the presence of a nitrogen source. Further evidence of this is the stimula-

TABLE 3. Effects of methane concentration on cellular incorporation of $^{14}CH_4$-C by *Nitrosococcus oceanus* and *Nitrosomonas europaea*

Organism	NH_4-N (ppm)	Ratio ^{14}C-cellular material/$^{14}CO_2$ at given CH_4 concn (mM)						
		1.19×10^{-5}[a]/ 1.38×10^{-5}[b]	1.19×10^{-4}/ 1.38×10^{-4}	1.19×10^{-3}/ 1.38×10^{-3}	5.95×10^{-3}/ 6.90×10^{-3}	1.19×10^{-2}/ 1.38×10^{-2}	5.95×10^{-2}/ 6.90×10^{-2}	1.19×10^{-1}/ 1.38×10^{-1}
Nitrosococcus oceanus	0	0.016	0.011	0.017	0.041	0.096	0.387	0.488
	10	0.008	0.009	0.006	0.011	0.010	0.149	0.757
Nitrosomonas europaea	0	0.389	0.426	0.394	ND[c]	1.133	ND	1.733
	10	0.207	0.214	0.223	ND	0.277	ND	0.316

[a] Methane concentration for *Nitrosococcus oceanus*.
[b] Methane concentration for *Nitrosomonas europaea*.
[c] ND, Not determined.

tion of methane oxidation by nitrite and nitrate. These data indicate an ammonium-stimulated co-oxidation of methane and ammonium. Although this co-oxidation is most active in the presence of ammonium, the oxidation of methane is not dependent upon the presence of ammonium.

The pH range for methane oxidation was quite wide, as for ammonium oxidation. The lower optimum pH for methane oxidation is interesting

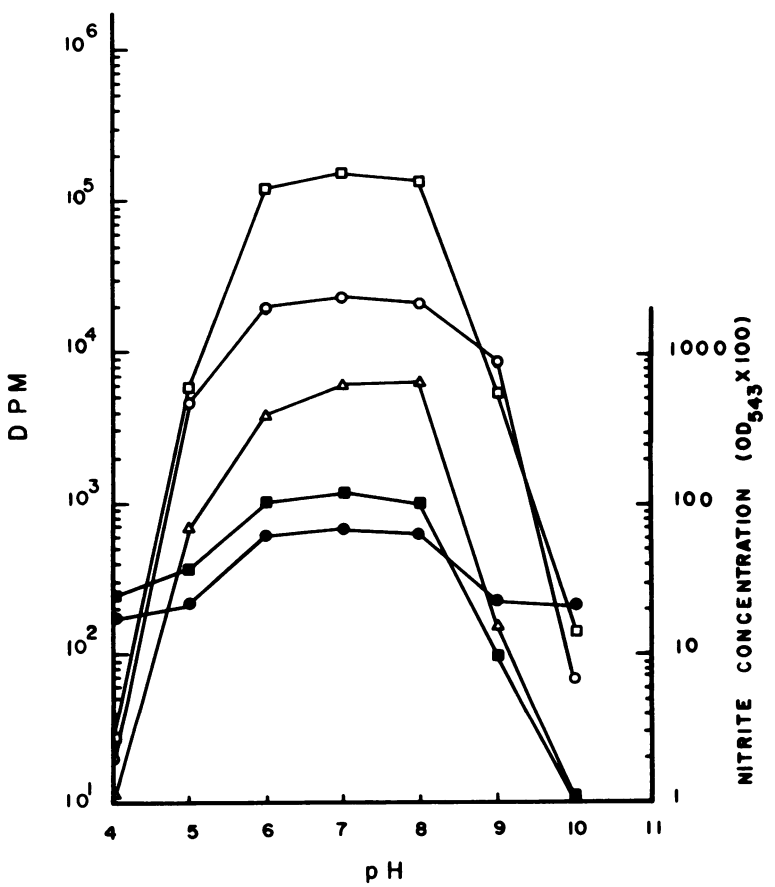

FIG. 4. Effects of pH on methane oxidation by *Nitrosococcus oceanus*. Activity is expressed as dpm of ^{14}C ml of standard inoculum^{-1} 24 h^{-1}. Symbols: (○) $^{14}CO_2$ produced, 0.0 ppm of NH_4-N (●) ^{14}C incorporated, 0.0 ppm of NH_4N; (□) $^{14}CO_2$ produced, 10 ppm of NH_4-N; (■) ^{14}C incorporated, 10 ppm of NH_4-N; (△) nitrite produced.

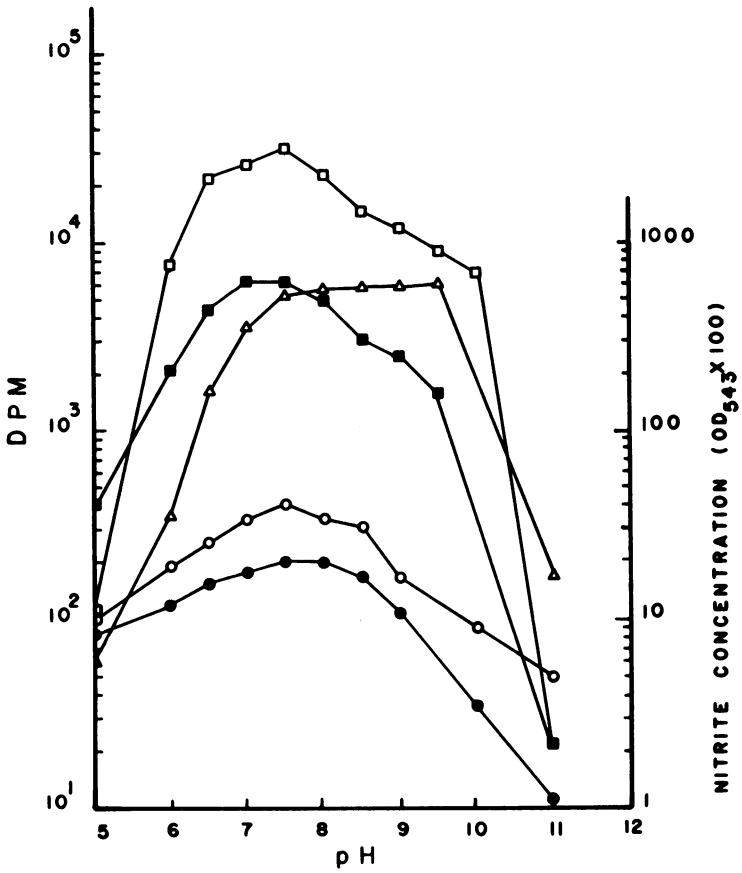

FIG. 5. Effects of pH on methane oxidation by *Nitrosococcus europaea*. Activity is expressed as dpm of ^{14}C ml of standard inoculum^{-1} 24 h^{-1}. Symbols: (○) $^{14}CO_2$ produced, 0.0 ppm of NH_4-N; (●) ^{14}C incorporated, 0.0 ppm of NH_4-N; (□) $^{14}CO_2$ produced, 10 ppm of NH_4-N; (■) ^{14}C incorporated, 10 ppm of NH_4-N; (△) nitrite produced.

since methane does not have a similar ammonia-ammonium shift with pH and its corresponding effect on activity as reported by Anthonisen et al. (1).

The oxidation of methane by *Nitrosomonas europaea* follows the rules for first-order kinetics. *Nitrosococcus oceanus* also follows this approach until a methane concentration of 1.19 × 10^{-2} mM in the presence of 10 ppm of ammonium. The continued increase in cellular incorporation and decrease in $^{14}CO_2$ production indicate a shift in the metabolic pathway. This is further demonstrated by the continued increase of the ^{14}C-cellular incorporation/$^{14}CO_2$ ratios for both organisms. This shift depends upon the organism and the presence or absence of ammonium and could represent a point at which the methane concentration in solution becomes high enough for the organism to stop using methane solely for energy to maintain itself and start building structural components. That the presence of 10 ppm of NH_4-N shifts this point to a higher value in *Nitrosococcus oceanus* is indicative of this. Another factor in this shift is that at

TABLE 4. Effects of carbonate concentration on cellular incorporation of $^{14}CH_4$-C in the presence of 10 ppm of NH_4-N

Organism	Ratio ^{14}C-cellular material/$^{14}CO_2$ at given carbonate concn (ppm)				
	0	10	50	200	500
Nitrosococcus oceanus	0.018	0.015	0.012	0.007	0.006
Nitrosomonas europaea	0.276	0.239	0.200	0.193	0.185

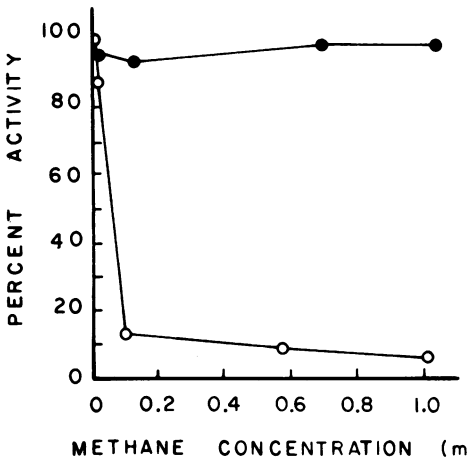

FIG. 6. Methane effects on ammonium oxidation by *Nitrosococcus oceanus* and *Nitrosomonas europaea*. Activity is expressed as percentage of nitrite formed in 12 h at 0.0-ppm CH_4 levels. Symbols: (○) *N. oceanus*; (●) *N. europaea*.

low methane concentrations ammonium oxidation appears to be responsible for at least 50% of the $^{14}CH_4$-C incorporation (Table 4) and that the uptake of $^{14}CO_3$ was not stimulated by the addition of methane in the absence of ammonium.

The rate of methane oxidation by ammonium oxidizers ranged from 2.2×10^{-2} μmol of CH_4 h^{-1} mg (dry weight) of cells^{-1} for *Nitrosococcus oceanus* in the absence of ammonium to 196.0×10^{-2} μmol of CH_4 h^{-1} mg (dry weight) of cells^{-1} for *Nitrosomonas europaea* in the presence of 10 ppm of NH_4-N. These rates indicate that methane may play a substantial role in the metabolism of these bacteria under certain conditions. The rates of methane oxidation are from 3.0 to 120% of the methane oxidation rate reported for the methane oxidizer *Methylosinus trichosporium*, the organism with the highest methane oxidation rate, and 10.1 to 389% of the rates for *Methylomonas agile*, the organism with the lowest methane oxidation rate as reported by Whittenbury et al. (13). These values demonstrate that the rate of methane oxidation by ammonium oxidizers is significant and may actually exceed that of the classical methane oxidizers under some conditions.

From an energetics viewpoint the oxidation of 1 mol of methane provides more energy than the oxidation of 1 mol of ammonium. It is probable that the co-oxidation of methane by ammonium oxidizers is a significant source of energy and cellular material under certain conditions, especially when the optimum pH for ammonium oxidation by *Nitrosomonas europaea* is 9.5. It is also possible that methane could serve as a source of energy when ammonium is limiting. It appears that the oxidation of methane is most likely not limited just to these organisms but seems likely to be a characteristic of all of the classical chemolithotrophic ammonium oxidizers. The ability of ammonium oxidizers to be able to switch from the oxidation of ammonium to methane and vice versa could be a survival mechanism when one or the other is not available as the energy source. Since methane is nearly always present in low concentrations, the survival advantage of being able to use either substrate is obvious. In situations where both ammonium and methane are present in nongrowth-sustaining concentrations, the combination of the two and co-oxidation by ammonium oxidizers may be able to supply the necessary carbon and energy requirements.

Attempts to grow the ammonium oxidizers on methane as the sole source of carbon and energy have failed even in the presence of 1.0 ppm of NH_4-N as evidenced by cell counts. These results are indicative of a difference between the ammonium- and methane-oxidizing bacteria.

Griffiths et al. (6) report that in their study of naturally occurring methane oxidation the proportion of CH_4 incorporated was low, indicating that methane was used as an energy source. This is suggestive of a role by ammonium oxidizers. They also report that there is a distinct difference between their results for a marine system and those reported for freshwater systems. Our data show that there is a distinct difference between marine and freshwater, estuarine, and soil ammonium oxidizers in the amount of CH_4 incorporated. With the exception of *Nitrosomonas* sp. strain 3S30, marine isolates incorporate significantly less $^{14}CH_4$-C than the other isolates. This discrepancy appears to be due in part to the relative carbonate concentration in freshwater and marine systems.

In aerobic sediments and water columns overlying methanogenic sediments, ammonium oxidizers could account for a significant portion of the methane oxidation. This type of environment is usually high in ammonium and other forms of inorganic nitrogen such that methane oxidation by ammonium oxidizers would be at an optimum. Harris and Hanson (7) in their work with organisms in Lake Mendota (Wisconsin) show that nitrite is confined to the part of the water column where rapid methane oxidation occurs. They also state that this indicates that since methanotrophs are known to oxidize ammonium (10, 13) methanotrophs may contribute significantly to the nitrogen cycle in Lake Mendota. This study indicates that classical ammonium oxidizers may be responsible for a portion of the methane oxidation and that the presence of high levels of nitrite helps to confirm this. The

oxidation of methane by *Nitrosomonas europaea* has been confirmed by Wood and Hyman (Soc. Gen. Microbiol. Annu. Meet., P2, 1982). The observations made in this study indicate that much more work needs to be done to characterize the physiology and role that ammonium oxidizers may play in the cycling of methane.

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