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Recovery of nitrification in marine bacteria following exposure to carbon monoxide or light

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ABSTRACT: Recovery of activity by 2 marine nitrifying bacteria, *Nitrosomonas cryotolerans* and *Nitrobacter* sp. Nb297, was monitored after exposure to light and/or carbon monoxide. *N. cryotolerans* recovered more rapidly after exposure to artificial light (25 W m^{-2}) than CO (11.4 µM). The addition of 100 µM NH_4^+ during the time of light or CO exposure allowed the cells to recover faster and decreased the difference observed between the inhibitory effects of light and CO. Cells exposed to sunlight recovered much more slowly than cells exposed to either CO or artificial light. The ammonium oxidizer *N. cryotolerans* when exposed to sunlight (2 h) recovered up to 35 % of its original ammonium oxidizing activity. Recovery increased to 57 % in the presence of additional substrate (100 µM NH_4^+). The nitrite oxidizer *Nitrobacter* sp. Nb297 did not show a noticeable recovery from sunlight whether substrate was added or not.

INTRODUCTION

In the oceans, chemolithotrophic nitrifying bacteria (ammonium and nitrite oxidizers) are subjected to periods of low nutrient availability (Morita 1982, Jones & Morita 1985). During these periods, nitrifying organisms may be subjected to additional stresses such as light (Müller-Neuglück & Engel 1961, Hooper & Terry 1974, Horrigan et al. 1981, Olson 1981a, b, Yoshioka & Saijo 1984, 1985, Shears & Wood 1985, Ward 1985) and CO (Hooper & Terry 1973, Suzuki et al. 1976, Jones & Morita 1984a, b). Olson (1981b) reported different patterns of photoinhibition for marine nitrifiers; ammonium oxidizers appeared to be less sensitive to light than nitrite oxidizers. Further, it was postulated that the primary nitrite maximum is a consequence of the differential inhibition observed (Olson 1981b). Photochemically formed CO is moreover a common feature in the oceans (surface concentrations of 2 to 100 nM; Conrad & Seiler 1980, 1985, Conrad et al. 1982). Cycling of nitrogen in the surface waters of the sea may therefore depend upon the ability of these organisms to recover from light and CO. Vanzella et al. (1989) showed that high substrate concentration benefits actively growing marine nitrifiers since it increased their tolerance to these inhibitors.

This paper examines the ability of marine nitrifying bacteria to recover from light and CO inhibition, with or without the addition of substrate. A better understanding of this is necessary to aid in answering questions dealing with the formation of the nitrite maximum and the ecological importance of these bacteria which represent a link between the carbon and nitrogen cycles in aquatic systems (Karl et al. 1984, Morita & Jones 1986).

MATERIALS AND METHODS

Cultures. The marine ammonium oxidizing bacterium *Nitrosomonas cryotolerans* (Jones et al. 1988), and the nitrite oxidizing bacteria *Nitrobacter* sp. Nb297 (provided by S. W. Watson) were grown in the dark, in 4 l chemostat culture units (0.14 d^{-1} dilution rate) equipped with automatic pH control that maintained a pH of 7.8 ± 0.05 by addition of 5 % K_2CO_3. Ammonium oxidizer medium contained 0.011 M of NH_4^+ as $(NH_4)_2SO_4$ and the salinity was adjusted to 30 ‰ using Instant Ocean Synthetic Sea Salts (Aquarium Systems, Inc., Mentor, Ohio; Jones & Hood 1980). Nitrite oxidizers were cultured in the same manner in the medium of Watson & Waterbury (1971). The concentration of NO_2^- as $NaNO_2$ was 0.029 M. Cultures were vigorously aerated, agitated by a magnetic stirring bar, and kept at a constant temperature of 25 °C.

Preparation of standard inoculum. Cells were harvested from 250 ml chemostat culture by centrifugation

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(6000 × *g*; 15 min; 5 °C), washed twice with filtered (Whatman GF/C, 1.2 µm) Sargasso Sea water (SSW), containing 0.02 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) pH 7.8, and then resuspended in 10 ml of SSW. Cell density was determined using a Petroff-Hauser counting chamber. From this suspension, 0.1 ml was inoculated into a 60 ml sterile serum bottle containing 25 ml of SSW. The final cell density was ca 5×10^5 cells ml^{-1} for the ammonium oxidizer and 1.1×10^6 cells ml^{-1} for the nitrite oxidizer. SSW used for the preparation of cell suspensions had been previously purged for 15 min with CH_4- and CO-free air to remove dissolved CO and CH_4. SSW was low enough in nutrients such that it did not interfere with the results (< 20 nM NH_4^+; < 10 nM NO_2^-; < 20 nM NO_3^-). Neither ammonium nor nitrite oxidation was detected in SSW.

All experiments described below were replicated and samples within each experiment were collected and analyzed in triplicate. All data points reported are mean values of the replicates in individual experiments and fell within a 95 % confidence interval. Statistical inferences were made using a 1-way analysis of variance (ANOVA), F values were determined and experimental values were found to be significantly different if p was < 0.01.

Exposure and recovery from CO inhibition. After inoculation, $(NH_4)_2SO_4$ was added to a final concentration of 0 or 100 µM NH_4^+. Bottles were sealed with serum stoppers and CO was injected into the headspace to give final concentrations of 11.4 µM (Schmidt 1979, Vanzella et al. 1989). Exposure times ranged from 1 to 3 h. Bottles were incubated in the dark at 25 °C on a rotatory shaker at 150 rpm. After exposure, cells were harvested by centrifugation, washed with SSW to remove CO and all traces of NH_4^+ and NO_2^-, and resuspended in SSW. From this suspension 1 ml was inoculated into 150 ml serum bottles containing 124 ml of NH_4^+-free medium. Final cell concentration was ca 4×10^5 cells ml^{-1} NH_4^+ as $(NH_4)_2SO_4$, was added to each bottle to achieve a final concentration of 10 µM. Bottles were sealed with silicon rubber stoppers and incubated in the dark on a rotatory shaker (150 rpm) at 25 °C for up to 24 h. At different time intervals 25 ml samples were removed from each bottle and assayed for nitrite production as an index of ammonium oxidation (Bendschneider & Robinson 1952) Recovery of *Nitrobacter* sp. from CO was not examined since these organisms were not significantly inhibited by any of the CO concentrations previously tested (Vanzella et al. 1989).

Exposure and recovery from light inhibition. Standard inocula of *Nitrosomonas cryotolerans* and *Nitrobacter* sp. were inoculated into screw-cap test tubes (25 × 117 mm) which contained 25 ml of SSW Substrate concentrations were either 0 or 100 µM of NH_4^+ for the ammonium oxidizer and either 0 or 2 µM of NO_2^- for the nitrite oxidizers. Tubes were incubated in a water bath at 25 °C under a daylight fluorescent light (Philips F30T12/DS/R). The temperature-controlled water bath was a flat-black painted box with circulating distilled water. Light intensity was measured with a LI-COR (model 1800) calibrated radiometer. Irradiance was determined to be ca 25 W m^{-2} at the surface. Tubes were lying horizontally 1 cm under the water surface in a rack placed in the water bath. Control tubes were wrapped in aluminium foil. In order to compare the effects of sunlight on NH_4^+ or NO_2^- oxidation, the water bath was placed under full sunlight. Sunlight irradiance was measured to be ca 628 W m^{-2}. After a 2 h exposure, cells were harvested and resuspended as described above. In the case of *Nitrobacter* sp., recovery studies were done using only sunlight, since artificial light has no effect (Vanzella et al. 1989).

RESULTS

Recovery of ammonium oxidation from CO and light inhibition by *Nitrosomonas cryotolerans*

After a 2 h exposure of the ammonium oxidizers to 11.4 µM CO or artificial light, cells showed a quick recovery of ammonium oxidation (Fig. 1A, B). Table 1 shows the percent recovery of ammonium oxidation after 24 h when cells were exposed to light or CO with

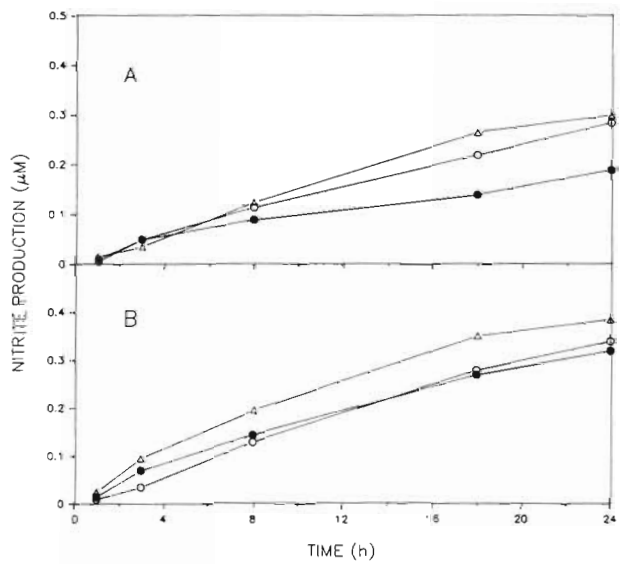

Fig. 1. *Nitrosomonas cryotolerans*. Recovery of ammonium oxidizing activity after 2 h exposure to artificial light or CO with either (A) 0 µM NH_4^+ or (B) 100 µM NH_4^+ during the exposure. (○) Illuminated cells; (●) 11.4 µM CO; (△) control. NO_2^- production is expressed at a density of 1×10^6 cells ml^{-1}. Light intensity was 25 W m^{-2}

Table 1. *Nitrosomonas cryotolerans*. Percent ammonium oxidation 24 h after exposure to artificial light (25 W m^{-2}) and CO, calculated using controls as 100 % recovery and at a density of 1×10^6 cells ml^{-1}

	Hours of exposure		
	1	2	3
CO (11.4 μM)	82 %	61 %•	52 %•
CO with 100 μM NH_4^+	88 %	84 %+	73 %•+
Full light	98 %	94 %	74 %•
Full light with 100 μM NH_4^+	81 %	91 %	78 %

• Indicates significantly different values within row, 1-way ANOVA, $p < 0.01$
+ Indicates significantly different values within column, 1-way ANOVA, $p < 0.01$

0 or 100 μM NH_4^+ added during exposure. Regardless of the time of exposure, the recovery from CO in the absence of ammonium was slower when compared to recovery from light. Since the CO concentration and light intensity used caused complete or almost complete inhibition of ammonium oxidation (Vanzella et al. 1989) it was assumed that the initial oxidation rates were close to zero.

After 24 h of recovery, the cells exposed for 1 or 2 h to artificial light had significantly greater oxidation rates ($p < 0.01$) than cells exposed for 3 h. Cells exposed to CO showed a decrease in percent recovery as the time of exposure increased ($p < 0.01$). Nevertheless, when exposed for 3 h, cells recovered at least half of their original activity after 24 h. Addition of 100 μM NH_4^+ during the time of CO exposure allowed the cells to recover activity significantly faster ($p < 0.01$), even in the case of cells exposed for 2 or 3 h (Table 1).

Recovery of ammonium oxidation following a 2 h exposure to sunlight (Fig. 2) was slower when compared to nitrification following exposure to artificial light. When 100 μM NH_4^+ was present during exposure to sunlight, recovery of nitrification after 24 h increased significantly (Table 2).

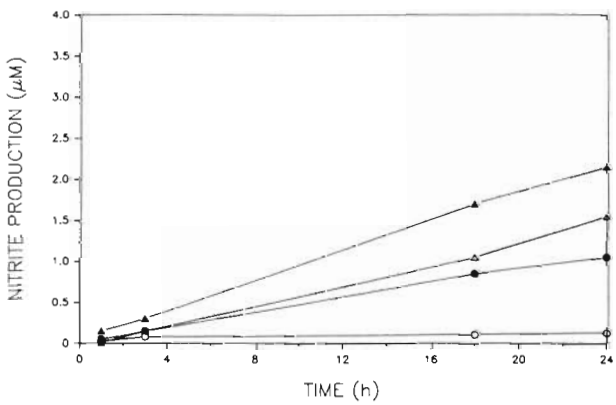

Fig. 2. *Nitrosomonas cryotolerans*. Recovery of ammonium oxidizing activity after 2 h exposure to sunlight. (○) Illuminated cells, 0 μM NH_4^+; (●) non-illuminated cells, 0 μM NH_4^+; (△) illuminated cells, 100 μM NH_4^+; (▲) non-illuminated cells, 100 μM NH_4^+. NO_2^- production is expressed at a density of 1×10^6 cells ml^{-1}. Light intensity was 628 W m^{-2}

Table 2. *Nitrosomonas cryotolerans* and *Nitrobacter* sp. Nb 297. Percent recovery of nitrification 24 h after exposure to sunlight (2 h exposure; 8 W m^{-2}), calculated using controls as 100 % recovery and at a density of 1×10^6 cells ml^{-1}

	Nitrosomonas	*Nitrobacter*
Full sunlight	35 %	3.0 %
Full sunlight with 100 μM NH_4^+ or 2 μM NO_2^-	57 %•	3.4 %

• Indicates significantly different values within column, 1-way ANOVA, $p < 0.01$

Recovery of nitrite oxidation from sunlight inhibition by *Nitrobacter* sp.

Recovery from full sunlight by nitrite oxidizers was very slow. After 24 h there was only 0.013 μM of NO_2^- oxidized (Fig. 3) compared to 1.63 μM of NO_2^- oxidized by the controls. Addition of 2 μM NO_2^- during the time of exposure did not significantly affect recovery. After 24 h, 0.015 μM of NO_2^- was oxidized by cells exposed to sunlight and 1.82 μM by cells kept in the dark.

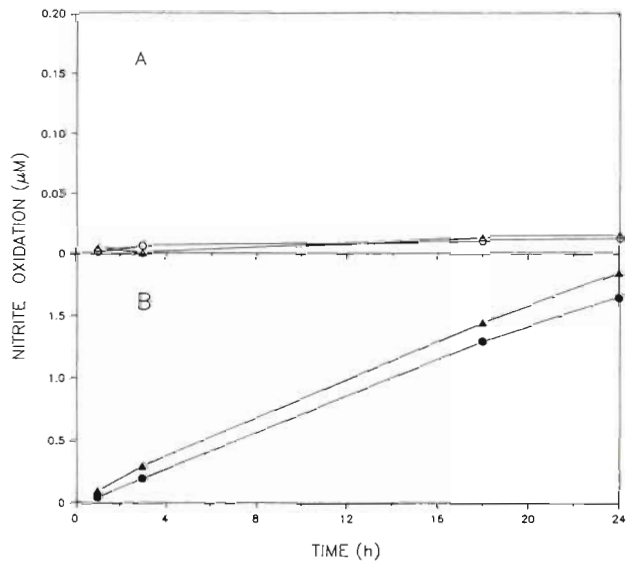

Fig. 3. *Nitrobacter* sp. Nb297. Recovery of nitrite oxidizing activity after 2 h exposure to sunlight. (A) Illuminated cells; (B) non-illuminated cells. (○, ●) 0 μM NO_2^-; (△, ▲) 2 μM NO_2^-. NO_2^- disappearance is expressed at a cell density of 1×10^6 cells ml^{-1}. Sunlight intensity was 628 W m^{-2}

DISCUSSION

Previous research has shown that ammonium and nitrite oxidizers can be photoinhibited (Müller-Neuglück & Engel 1961, Hooper & Terry 1974, Horrigan et al. 1981, Olson 1981a, b, Yoshioka & Saijo 1984, 1985, Shears & Wood 1985, Ward 1985, Vanzella et al. 1989). However, recovery has not been extensively studied, especially in regards to recovery from CO exposure.

Hooper & Terry (1974) reported a 90% recovery of NH_4^+ oxidation by *Nitrosomonas europaea,* 6 h after illumination for 45 min with a Kodak Carrousel 500 DEK Lamp. Horrigan et al. (1981) and Yoshioka & Saijo (1984), on the other hand, found recovery from photoinhibition to take more than 7 d after being irradiated for 8, 12 or 16 h by cool white fluorescent light. Test organisms were isolates from natural seawater samples for the former report; and strain H-1 (probably *Nitrosospira* sp.) or strain O-1 (probably *Nitrobacter* sp.) for the latter study. Exposure time, light quality, differences in strains and light intensity are some of the factors that could account for the contrasting results (all except Yoshioka & Saijo used intensities ≥ 36 W m^{-2}).

A protective effect was observed when *Nitrosomonas cryotolerans* was illuminated for 2 or 3 h in the presence of 100 µM NH_4^+. Ammonia also protected these cells when exposed to CO (Tables 1 and 2). The photochemical production of CO in seawater (Wilson et al. 1970), in addition to its inhibitory effects on ammonium oxidation (Jones & Morita 1984a, b, Vanzella et al. 1989), make the exact mechanism of photoinhibition of ammonium oxidizers complex. It may be that CO formed during illumination causes inhibition, instead of, or in addition to, the possible oxidation of cytochrome c_{554} (Suzuki et al. 1974, Tsang & Suzuki 1982); or that light itself affects other cellular components, probably at the membrane level.

Müller-Neuglück & Engel (1961) reported that *Nitrobacter winogradsky* was reversibly inactivated by relatively low light intensities (4900 lx; ca 19 W m^{-2}). In contrast, previous research in our laboratory showed *Nitrobacter* sp. Nb297 to be insensitive to artifical light (≤ 25 W m^{-2}) but found that sunlight (628 W m^{-2}) decreased nitrite and ammonium oxidation to 15% and 7%, by *Nitrobacter* and *Nitrosomonas cryotolerans* respectively, after 2 h of illumination (Vanzella et al. 1989). The data here shows a slower recovery from sunlight for the nitrite oxidizer (Fig. 3) when compared to the recovery of the ammonium oxidizer (Fig. 2). After 24 h *Nitrobacter* showed only a 3% recovery of activity compared to the controls; even the presence of 2 µM NO_2^- did not accelerate the recovery process. *N. cryotolerans,* on the other hand, showed a significant recovery of activity after sunlight exposure, which markedly increased if 100 µM NH_4^+ was added (Table 2). Earlier research by Schön & Engel (1962) had depicted light as a lethal agent to *Nitrosomonas europaea,* if cells were illuminated for a long period with sufficient light intensity. On the other hand, Johnstone & Jones (1988) have shown that light inhibition of long-term starved *N. cryotolerans* only becomes lethal in the absence of substrate.

Recovery from CO was slower for all lengths of exposure than recovery from artificial light when no ammonium was present during the time of irradiation (Table 1). This possibly reflects the fact that irradiation by artificial light never caused a 100% inhibition while a CO concentration of 11.4 µM completely inhibited NH_4^+ oxidation (Vanzella et al. 1989).

The fact that *Nitrosomonas cryotolerans* recovered its ability to oxidize ammonium more quickly after exposure to a CO concentration at which NH_4^+ oxidation was completely inhibited indicates that CO is not lethal to the cells (Fig. 1A, B) and that CO inhibition is reversible. Apparently, once the higher affinity substrate, CO, (Jones & Morita 1983) is removed, cells almost immediately start oxidizing NH_4^+, their main substrate, and regenerating the affected cellular component(s). In this respect, Johnstone & Jones (1988) reported long-term starved *N. cryotolerans* as insensitive to CO even in the absence of substrate.

Fig. 1A, B corroborate the fact that in the presence of low substrate concentration, the inhibitory effect of CO is increased. The protective effect of NH_4^+ on CO inhibition was observed for all exposure times. Some of the nitrite production detected in cells exposed to light and CO with 100 µM NH_4^+ may be due to the presence of ammonium or hydroxylamine as a carryover even after cells were washed, but this effect was minimal and was also represented in the controls.

Translation of laboratory results to natural systems is always difficult. Nevertheless, given the distinct responses achieved with pure cultures of both organisms, it could be assumed that in natural systems where these bacteria are exposed to sunlight, the nitrite oxidizers are severely (non-recoverably) inhibited while ammonium oxidizers are inhibited but are capable of a fast recovery We believe that these recovery experiments give further support to the suggestion that different mechanisms of light and CO inhibition exist for the ammonium and nitrite oxidizers (Olson 1981b, Vanzella et al. 1989). But whether this is actually the case needs to be established by field studies.

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