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Combined autoradiography and immunofluorescence for estimation of single cell activity by ammonium-oxidizing bacteria[1,2]

Abstract—Immunofluorescence and $^{14}CO_2$ autoradiography were used for simultaneously enumerating and assaying the autotrophic activity of ammonium-oxidizing bacteria in seawater. Relative activity ($^{14}CO_2$ assimilation as measured by autoradiography) and abundance were measured in simulated in situ incubations at seven stations in the primary NO_2^- maximum region of the Northeast Pacific Ocean. More than 10^4 cells·liter^{-1} were present; relative activity often showed a peak near the surface and an increase in the NO_2^- max region below the photic zone. The method permits assessment of individual cell activity; most cells at all depths were active in CO_2 assimilation, usually at low and quite variable levels. There were no differences in relative activity between samples incubated under simulated in situ conditions and in the dark.

Relative activity was positively correlated with the abundance of ammonium-oxidizing bacteria, temperature, total dark CO_2 assimilation (as measured by liquid scintillation counting of replicate samples), and pheopigment concentration, and negatively correlated with oxygen concentration.

Microbial nitrification contributes to the formation and maintenance of the primary nitrite maximum common in coastal and oceanic waters (Olson 1981*a*,*b*; Ward et al. 1982). The autotrophic ammonium- and nitrite-oxidizing bacteria involved are present at concentrations of 10^3–10^5 cells·liter^{-1} (Ward 1982). The in situ nitrification rates of these bacteria have been measured with ^{15}N substrates (e.g. Hattori and Wada 1971; Miyazaki et al. 1973, 1975). However, ^{15}N is not an optimal tracer for microbial activities; its use involves samples as large as 4 liters, long incubations, substantial substrate enrichment, and extensive processing.

These disadvantages can be avoided by using $^{14}CO_2$ as a tracer for measuring nitrifying activity in terms of CO_2 assimilation (Billen 1976; Somville 1978). Because ammonium oxidizers are obligate autotrophs (Hooper 1969), their growth depends directly on nitrogen oxidation. However, the N-serve [2-chloro-6-(trichloromethyl)pyridine], a selective inhibitor of nitrification, used in this technique is probably not a reliable measure of either the autotrophic activity or the nitrification activity of nitrifying bacteria in environmental samples because the ratio of carbon assimilation to nitrogen oxidation varies under different conditions (Gundersen 1966; Glover unpubl.) and N-serve affects carbon assimilation of other bacteria and phytoplankton (Ward unpubl. data).

Fliermans and Schmidt (1975) combined $^{14}CO_2$ autoradiography with cell counts by immunofluorescence to study nitrite-oxidizing bacteria in soils and found that the activity of the nitrifiers (percent active cells on the autoradiograph) did not parallel cell growth (cell counts) except during the early exponential phase of a batch culture. They did not measure nitrite oxidation but did provide indirect evidence that cell number, carbon assimilation, and nitrogen oxidation by nitrifying bacteria are not correlated in simple ratios.

I report here the use of combined autoradiography-immunofluorescence to study ammonium-oxidizing bacteria in the nitrite maximum region of the Northeast Pacific Ocean off the Washington coast. All field data were collected on Cruise W8009C of the RV *Wecoma* in September–October 1980.

Samples from the upper 50–100 m (6–8 depths per cast) were collected in 5-liter Niskin bottles mounted on a rosette sampler together with a CTD, oxygen probe, and transmissometer and filtered through 10-μm mesh Nitex screen. Pyrex bottles (125 ml) were washed with detergent, acid-rinsed (10% HCl), and then rinsed exhaustively with distilled water. After three seawater

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rinses each bottle was filled with 100 ml of the sample and isotope solution (26.26 μCi·ml^{-1} as $NaH^{14}CO_3$) was added (0.5 ml or 1.0 ml per bottle).

Three light and three dark bottles for each depth were incubated for 24 h under simulated in situ (SIS) conditions in running seawater incubators on deck. For light attenuation bottles were put into Plexiglas tubes covered with nickel neutral density screens (Perforated Products, Inc.) calibrated with a 4-π collector (Biospherical Instruments, Inc.). After incubation, the contents of one bottle from each treatment were filtered for liquid scintillation counting (LSC). The other two were emptied into 250-ml medicine bottles containing 2 ml of Formalin (sodium acetate buffered to neutral pH and prefiltered) or 4 ml of 25% glutaraldehyde (sodium bicarbonate buffered to pH 9.5 and filtered just before the cruise for maximum activity). Thirty minutes later, replicate subsamples (5–20 ml) from the preserved samples were individually filtered by gentle (25 mm of Hg) vacuum onto 0.45-μm nominal pore size Sartorius filters (25 mm, cellulose nitrate). Each filter was rinsed twice with filtered seawater and twice with ammonium formate (0.6 M) to remove salts, placed in individual plastic pill boxes, and stored in a desiccator.

Autoradiographs were prepared from the stored filters by the method of Brock and Brock (1968), as modified by Paerl (1974) and Carlucci (A. F. Carlucci pers. comm.). The central portions of the dried filters were cut out and attached to ethanol-cleaned 25- × 75-mm glass slides with tiny drops of white glue, cleared over fuming acetone, and placed in order in black plastic slide boxes containing silica gel desiccant.

An emulsion dipping method was used to prepare autoradiographs. In total darkness slides were dipped individually into Kodak NTB-2 nuclear track emulsion (diluted 1:1 with sterile-filtered distilled water), allowed to dry for 30 min, and returned to their boxes, which were sealed with black electrical tape and stored at room temperature for 3 weeks to allow exposure of the emulsion. Autoradiographs were developed by immersing in Acufine developer for 1.5 min, distilled water for 10 s, and finally in Rapid Fixer (Kodak) for 4 min. Slides were then rinsed in running distilled water for 5 min and allowed to dry at 27°C.

The indirect immunofluorescence method (Ward and Perry 1980) was adapted for staining through the emulsion layer on autoradiographs prepared as above. A ring of clear fingernail enamel on top of the emulsion served as a well for the addition of stain solutions. The slides in a holder were immersed in phosphate-buffered saline solution (pH 7.6: Ward and Perry 1980) and after 5 min removed and placed in humidified chambers, supported at each end to prevent marring of the emulsion. Thirty microliters of each specific antiserum against *Nitrosococcus oceanus* and *Nitrosomonas marina* (Ward and Perry 1980; Ward 1982) were dropped into the ring of nail enamel by automatic pipet, filling the filter area to its rim. After incubation for 12 h at room temperature, the excess antiserum was rinsed off with phosphate-buffered saline and the slides immersed for two 5-min washes in fresh phosphate-buffered saline. Slides were then returned to the humidified chambers and incubated for 12 h with 50 μl of fluorescein-isothiocyanate-conjugated sheep antirabbit IgG (diluted 1:1,000 with sterile phosphate-buffered saline, Miles Yeda Ltd.). Excess fluorescent antibody was removed by rinsing with phosphate-buffered saline, followed by two 5-min washes in phosphate-buffered saline and a last 5-min wash in distilled water, and the slides again dried at 27°C. A drop of mounting solution (carbonate buffer pH 9.5 and glycerol 1:1 v/v) was placed over the filter area and a cover slip emplaced and sealed with fingernail enamel.

Autoradiographs were examined with a Zeiss Universal microscope. Transmitted and epifluorescent illumination was used simultaneously [the epifluorescent system with a 100-W tungsten halogen lamp, a blue excitation filter (450–500 nm) and a yellow-green fluorescence barrier filter (528 nm)]. The slide was scanned by epifluorescence (the transmitted beam obscured with an opaque cover) until a stained cell was found. With transmitted light, the stained cell was outlined in green and the developed grains around it were orange. One hundred fields

Table 1. Stations for autoradiography experiments on Cruise W8009C.

Station	1980	Location	Total depth of water column (m)	Activity added (μCi·ml^{-1})	Length of incubation (h)
37	27 Sep	47°07.0N, 124°22.5W	51	0.243	12
68	29 Sep	47°13.7N, 124°57.6W	445	0.243	24
131	5 Oct	47°07.7N, 124°57.9W	190	0.121	24
142	8 Oct	47°13.8N, 124°30.0W	65	0.121	24
143	8 Oct	47°07.2N, 124°29.0W	68	0.121	24
147	9 Oct	47°20.2N, 124°46.4W	180	0.121	24
155	11 Oct	47°06.8N, 124°40.6W	106	0.243	24

were counted. Each stained cell was centered in a square (400 squares per field) and all grains in that square were considered associated with it. An average background value for each filter was determined by counting all the grains in a grid area from a portion of filter with no cells present and dividing by 400. The relative activity (RA) of individual cells, calculated as the number of grains per cell minus the number of grains per square background is considered to be an index of CO_2 fixation on a single cell basis. It is not an absolute measurement of CO_2 fixation, was not calibrated to yield absolute values, and cannot be used directly to calculate chemosynthetic production.

Only a few ammonium-oxidizing cells were found on each filter; replicate filters were counted where possible. *Nitrosomonas* and *Nitrosococcus* were identified and distinguished as described by Ward (1982). The specificity of the sera has been extensively tested (Ward and Perry 1980; Ward 1982) to minimize the chance that nonnitrifying organisms might be stained.

Hydrographic and biological data were collected as described elsewhere (Postel et al. unpubl.). Ammonium, nitrite, and nitrate were assayed by autoanalyzer techniques, chlorophyll *a* and pheopigments determined fluorometrically (Lorenzen 1966). Oxygen was assayed in discrete samples by the Carpenter (1965) modification of the Winkler method. All analyses were completed at sea.

Because the counts made here (developed grains, numbers of cells) follow a Poisson distribution, while the normal distribution may be more appropriate to other variables, I used nonparametric statistical analyses (Tate and Clelland 1957); specific tests are noted below.

The abundance and relative activity of two species of ammonium-oxidizing bacteria were measured at the stations listed in Table 1. Total abundances of ammonium oxidizers were about 10^4 cells·liter^{-1}. *Nitrosomonas* was always more abundant than *Nitrosococcus* ($P < 0.05$), as in several other marine environments (Ward 1982). In general, the two species showed similar depth distributions within stations.

Relative activities of the ammonium-oxidizing bacteria for dark and SIS light incubation conditions are shown in Figs. 1–3; distributions of RA for the two species were similar within stations. The value of the index did not differ significantly between the two (Wilcoxon signed rank, $P > 0.125$). Abundances and relative activity at each depth multiplied to produce a total relative activity index, representing a relative measure of the total CO_2 assimilation by nitrifiers at each depth, are also shown. As expected, the depth distributions of total relative activity for the two species are similar; however, due to its greater abundance, total RA of *Nitrosomonas* exceeded that of *Nitrosococcus*.

No significant difference (Wilcoxon signed rank) in RA between SIS light and dark treatments was found at any station (P always > 0.125). Because the RA measurements were obtained by observation of individual cells, they can also be used to investigate the degree of uniformity of ac-

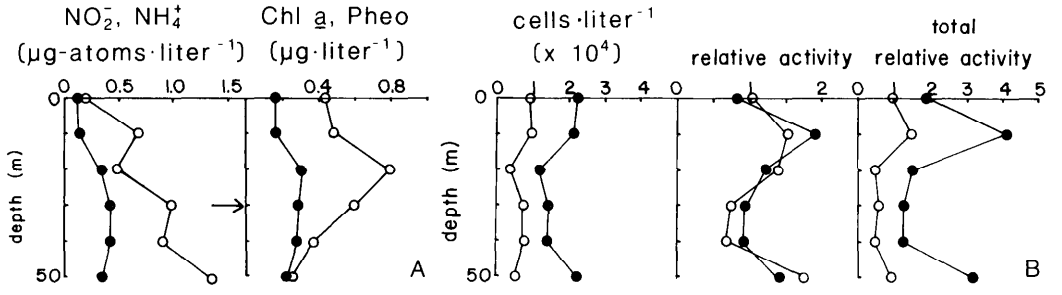

Fig. 1. A. Nutrient and pigment distributions at station 68. Nitrite and pheopigments—●; ammonium and Chl *a*—○. Arrow designates 1% light depth. B. Abundance and activity results for station 68. *Nitrosomonas*—○; *Nitrosococcus*—●. Only dark incubations were performed at this station.

tivity among cells at each depth: frequency distributions of RA among *Nitrosomonas* cells at station 147 are shown in Fig. 4. The Kolmogorov-Smirnov test was used to test for differences in the distribution of relative activity between dark and SIS samples at all photic zone depths at stations 142, 143, 147, and 155. At three stations, differences were not significant; significant differences were detected only at station 143 for *Nitrosococcus* at 20 m ($P < 0.01$) and for *Nitrosomonas* at 25 m ($P < 0.05$). In these two cases the difference is due to the higher number of cells from SIS samples in the higher activity range. Indeed, for this station, average relative activity in the SIS samples appeared to exceed that in dark samples at depths near the 1% light level (Fig. 2). However, this result is not representative of the stations investigated. As shown in Fig. 4, most cells had low RA, although in almost all cases >50% of the cells were active. Overall, the most active cells had RA up to 7 times greater than the least active cells at the same depth.

Fliermans and Schmidt (1975) used the

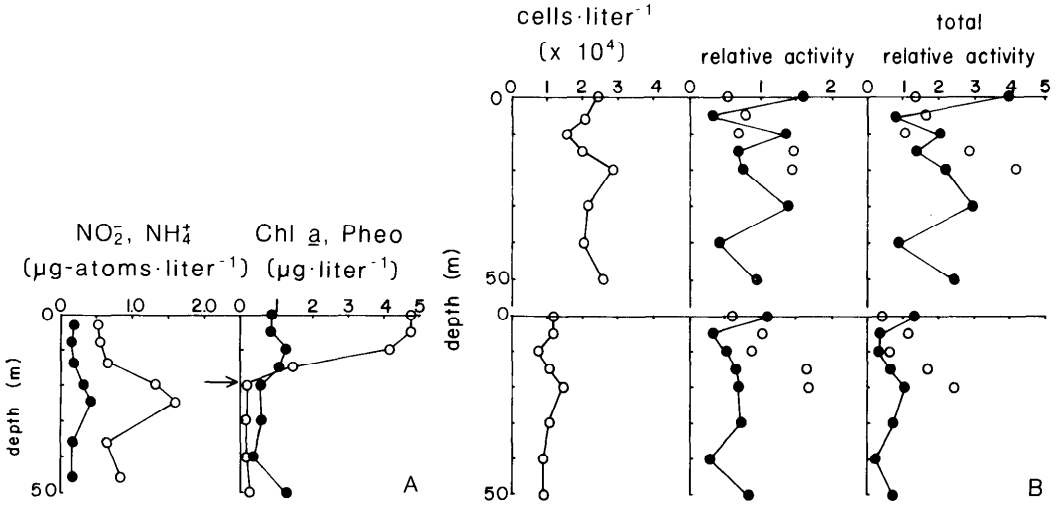

Fig. 2. A. As Fig. 1, but at station 143. B. Abundance and activity results for station 143. Top—*Nitrosomonas*; bottom—*Nitrosococcus*; ○—SIS samples; ●—dark samples.

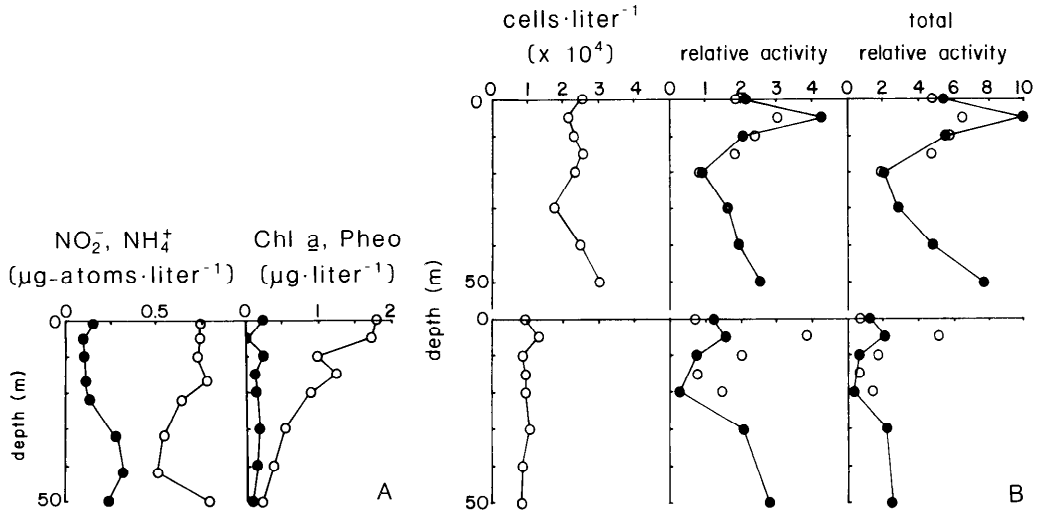

Fig. 3. A. As Fig. 1, but at station 147. B. As Fig. 2, but for station 147.

percentage of active cells as an index of CO_2 assimilation activity in their initial autoradiography work with *Nitrobacter*. This index requires only that the presence or absence of developed grains associated with stained cells be noted. I expressed my data in this way and used the Wilcoxon signed rank test to compare SIS and dark values at all photic zone depths in the four stations for which there was sufficient replication. Again, no differences in activity were detected between dark and SIS samples.

I used nonparametric correlation analysis to investigate relationships among relative activity measurements and other biological oceanographic variables. Kendall's tau was computed for the combined data for each depth at all seven stations for abundance of *Nitrosomonas* and *Nitrosococcus*; RA for each genus in SIS light and dark; oxygen concentration, nitrite, ammonium, chlorophyll, and pheopigment concentrations; temperature; CO_2 assimilation by the total natural population (LSC) in SIS light and dark; and percent incident light intensity. The only significant correlates found were between RA of the two species in the light ($P < 0.01$) and in the dark ($P < 0.01$). RA in the light was related to RA in the dark only at the $P < 0.10$ level. There were no significant correlations with any of the nutrients or other biological variables. When the seven stations were analyzed separately to avoid grouping data from stations which might have quite different nitrification or phytoplankton regimes (Table 2), however, correlations between RA and several other variables were found.

Recent evidence (Olson 1981*b*; Horrigan et al. 1981; Ward et al. 1982) suggests that nitrification in the upper layer of the ocean is at least partially controlled by light intensity. The biochemical basis for light inhibition of nitrifiers has been demonstrated (Bock 1978) and inhibition of nitrogen oxidation has been shown in enrichment cultures of ammonium- and nitrite-oxidizing bacteria collected from surface waters (Horrigan et al. 1981). SIS and dark incubations were performed here to test whether light affected CO_2 assimilation activity by nitrifiers; no differences were found. If all nitrifier cells in the photic zone were light-inhibited, then insufficient recovery time for the dark samples could explain this: several days were necessary for laboratory enrichment cultures held for several weeks on a 16:8 L:D cycle to recover and begin nitrite production (Horrigan et al. 1981).

Phytoplankton in the bottles would be expected to show major differences in activity in the SIS and dark treatments. Artifacts

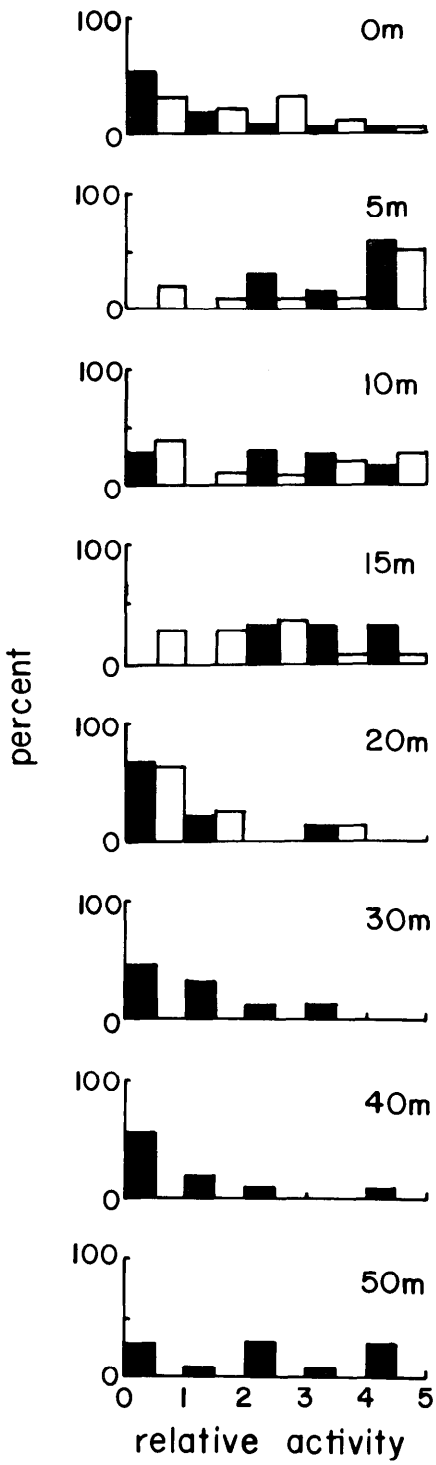

Fig. 4. Frequency distribution of relative activity among single cells of *Nitrosomonas* counted at station 147. Dark bars—dark incubated samples; open bars—SIS incubated samples.

Table 2. Correlations (Kendall's tau) of biological and environmental variables with relative activity (SIS or dark samples).

Correlate	Station	τ
Abundance of ammonium-oxidizing bacteria	68	0.64
	131	0.69*
	143	0.76†
	147	0.67
Nitrite concn	142	0.87*
	143	0.61
	155	0.65
Pheopigment concn	142	0.87†
	143	0.74†
	147	0.69*
	155	0.71*
	68	−0.87†
	131	−0.76†
Total dark CO_2 assimilation (LSC)	131	0.96†
	142	0.97†
	143	0.94†
	147	0.88†
Temp	131	0.89†
	143	0.78†
	147	0.88†
Oxygen concn	131	−0.79†
	147	−0.65*

* $P < 0.05$.
† $P < 0.02$.

due to activity of the natural mixed population or to methodological biases were investigated by testing for correlations among background, activity of isotope added to the incubation bottles, total CO_2 assimilation (LSC) of the incubated samples, and total number of grains per cell as well as relative activity per cell (Table 3). Each autoradiograph was considered a single case ($n = 175$) instead of each depth as in Table 2 ($n = 49$).

Background grain development was significantly correlated with raw counts of grains per cell for both *Nitrosomonas* and *Nitrosococcus* and with relative activity for *Nitrosomonas*. When the Wilcoxon signed rank was used to compare the background level of SIS and dark samples, the difference was significant only at station 131 ($P < 0.05$). Total CO_2 assimilation (LSC) was much greater in the SIS bottles than in the dark, due to photosynthetic CO_2 assimilation by phytoplankton, but no artifact associated with this disparity can be distinguished.

Table 3. Methodological correlates with relative activity.

Correlate 1	Correlate 2	
Specific activity	Grains/cell* (*Nitrosomonas*)	ns†
	Grains/cell (*Nitrosococcus*)	ns
	Background	ns
	RA‡ (*Nitrosomonas*)	ns
	RA (*Nitrosococcus*)	ns
CO_2 assimilation (LSC) SIS and dark combined	Grains/cell (*Nitrosomonas*)	ns
	Grains/cell (*Nitrosococcus*)	ns
	Background	ns
	RA (*Nitrosomonas*)	ns
	RA (*Nitrosococcus*)	ns
Background	Grains/cell (*Nitrosomonas*)	0.001
	Grains/cell (*Nitrosococcus*)	0.001
	RA (*Nitrosomonas*)	0.001
	RA (*Nitrosococcus*)	ns

* Raw counts of grains associated with each cell.
† Not significant ($P \gg 0.05$).
‡ Relative activity (raw counts − background).

Except possibly in unusual environments (Horrigan 1981), the contribution by chemoautotrophs to the total dark CO_2 assimilation of a mixed natural population (denoted here as LSC) is probably negligible. In fact, LSC measurements have been used to assess total heterotrophic production (Sorokin 1961). Although the interpretation of dark CO_2 assimilation is still unclear, LSC must represent some measure of nonphotosynthetic dark community metabolism. Although not expected a priori there was a significant positive correlation between this metabolism and CO_2 assimilation by nitrifiers at four of seven stations (*see Table 2*). This relationship appears to break down below the photic zone, where RA increases sharply while LSC is reduced to minimal levels. This pattern is in agreement with the assumption that dark-bottle $^{14}CO_2$ incorporation is due to heterotrophic processes which show correlations with photosynthetic activity in surface waters.

Pheopigment concentration was positively correlated with both LSC and RA (Table 2). Accumulations of pheopigments may correspond to increased levels of zooplankton excretion and perhaps to increased heterotrophic activity in associated organic debris. Decomposition of this material would release ammonium which could enhance nitrifier activity (RA) as well as heterotrophic activity (LSC). Microzones of high ammonium would not be measured by the standard nutrient analysis and might turn over very fast.

Nitrite concentration had a significant positive correlation with relative activity at one station (Table 2). Since nitrite is the end product of ammonium oxidation, this accumulation may be expected where high ammonium oxidation rates occur, but that increased ammonium oxidation rates are associated with high relative activity has not been established.

At two stations, oxygen concentration occurred as a negative correlate with RA, implying lower RA at higher oxygen concentration. This result is in agreement with the observation from culture work implying optimal nitrification efficiency under microaerophilic conditions (Gundersen 1966; Carlucci and McNally 1969) and with the suggestion by Cohen and Gordon (1978) that nitrification is favored under low oxygen conditions in the eastern tropical North Pacific.

Ammonium concentration was not correlated with RA at any station and nitrite at only one. These results imply that concentration of substrate and product in the bulk medium are not good indicators or predictors of activity (biological reaction rates). The methods I used to count and measure the activity of individual cells did not distinguish the distribution of the bacteria as free, attached, or perhaps associated with flocculent suspended material. Nitrifying bacteria in the water may exist under very localized favorable conditions so that the methods I used to assess environmental correlates were on the wrong scale. Instead, relative activity appeared more correlated with variables often associated with phytoplankton activity [pheopigment concentration, SIS, and dark assimilation (LSC)] and consistently showed a maximum at surface or

shallow subsurface depths, an intermediate minimum around the bottom of the photic zone, and an increase below the photic zone.

A peak in nitrifying activity is not expected in the photic zone due to light inhibition of nitrifying bacteria (*see above*). ^{15}N-ammonium oxidation rates are nearly negligible under photic zone conditions in the region of the primary nitrite maximum off southern California (Olson 1981*a*; Ward et al. 1982) and in the NE Pacific off the coast of Washington (Ward et al. in prep.). My data thus imply a high CO_2 assimilation activity (in both SIS and dark samples) where ammonium oxidation activity is expected to be minimal; i.e. CO_2 assimilation and ammonium oxidation may be largely uncoupled in the photic zone but more tightly coupled below the photic zone where ammonium oxidation activity is expected to be maximal (Olson 1981*b*).

Due to the low energy yield of the nitrification reactions, nitrifying bacteria can influence the oceanic nitrogen cycle to a degree disproportionate with their relatively low cell numbers; i.e. a great deal of nitrogen must be oxidized to produce relatively little cell growth. Earlier results (Ward et al. 1982) showed that although numbers of nitrifying bacteria were low and relatively constant through the water column, their rates of ammonium oxidation varied by a factor of 10–100. The coupling between nitrogen oxidation and cell growth is highly variable. If, on the other hand, relative activity as measured by autoradiography does give a relative measure of CO_2 assimilation, relative activity and cell numbers should be directly related at least on a time scale of generation times for the bacteria. This expectation is borne out by the significant positive correlation observed between these two measurements in this data set for two stations (Table 2).

These experiments were designed to measure the autotrophic activity of ammonium-oxidizing bacteria in the upper water, including the subsurface primary nitrite maximum. However, neither the relative activity nor the abundance of ammonium-oxidizing bacterial cells was correlated to the nitrite concentration in the water. Such a simple and direct correlation might not be expected for several reasons. RA does not measure nitrogen oxidation, the latter being the process directly involved in formation of the nitrite maximum. The primary nitrite maximum is a long term predictable feature in this region. Olson (1981*b*) has presented a time-dependent model for the formation of the maximum which assumes constant bacterial numbers with depth and constant light and substrate conditions over the period of nitrite accumulation. None of these assumptions is valid for my data set. For example, depth of the nitrite maximum and values of other biological variables changed significantly within a week between repeat visits to station 155. Thus, time scales relevant to features like the nitrite maximum may be different from the scales relevant to variations in the RA of individual cells as measured here.

In my limited data set, the distribution of autotrophic activity of nitrifying bacteria, as measured by autoradiography, is correlated with other biological variables in the upper photic zone, but diverges below that. High RA is correlated with high cell numbers of nitrifying bacteria, with lower oxygen concentrations, and with high pheopigment concentrations. Relative activity shows two peaks, one in the photic zone and one below it. The combined autoradiography-immunofluorescent approach, although time-consuming and somewhat complex, provides information on single cell activity that is not otherwise available.

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Quantitative grain density autoradiography and the intraspecific distribution of primary productivity in phytoplankton

Abstract—Analysis of a method of grain density autoradiography demonstrates that reliable measurements of the primary productivity of individual phytoplankton species can be obtained with this technique. Grain density autoradiography is particularly useful for providing an estimate of the intraspecific distribution of primary productivity. As an example, the productivity distribution of the marine diatom *Chaetoceros curvisetus* became positively skewed during a period of population decline.

Initial studies of quantitative grain density autoradiography used to estimate the primary productivity of algae at the species level were based on grain-counting techniques (Brock and Brock 1968; Maguire and Neill 1971; Watt 1971; Stull et al. 1973; Gutel'makher 1973). Knoechel and Kalff (1976*a*) discussed several potential problems inherent in early methods of grain density autoradiography and suggested that track counting was a superior technique (Knoechel and Kalff 1976*b*). Paerl and Stull (1979) demonstrated that equivalent data can be obtained with either method, but