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Ammonia-Oxidizing Bacteria in a Chloraminated Distribution System: Seasonal Occurrence, Distribution, and Disinfection Resistance

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Nitrification in chloraminated drinking water can have a number of adverse effects on water quality, including a loss of total chlorine and ammonia-N and an increase in the concentration of heterotrophic plate count bacteria and nitrite. To understand how nitrification develops, a study was conducted to examine the factors that influence the occurrence of ammonia-oxidizing bacteria (AOB) in a chloraminated distribution system. Samples were collected over an 18-month period from a raw-water source, a conventional treatment plant effluent, and two covered, finished-water reservoirs that previously experienced nitrification episodes. Sediment and biofilm samples were collected from the interior wall surfaces of two finished-water pipelines and one of the covered reservoirs. The AOB were enumerated by a most-probable-number technique, and isolates were isolated and identified. The resistance of naturally occurring AOB to chloramines and free chlorine was also examined. The results of the monitoring program indicated that the levels of AOB, identified as members of the genus *Nitrosomonas*, were seasonally dependent in both source and finished waters, with the highest levels observed in the warm summer months. The concentrations of AOB in the two reservoirs, both of which have floating covers made of synthetic rubber (Hypalon; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.), had most probable numbers that ranged from <0.2 to >300/ml and correlated significantly with temperature and levels of heterotrophic plate count bacteria. No AOB were detected in the chloraminated reservoirs when the water temperature was below 16 to 18°C. The study indicated that nitrifiers occur throughout the chloraminated distribution system. Higher concentrations of AOB were found in the reservoir and pipe sediment materials than in the pipe biofilm samples. The AOB were approximately 13 times more resistant to monochloramine than to free chlorine. After 33 min of exposure to 1.0 mg of monochloramine per liter (pH 8.2, 23°C), 99% of an AOB culture was inactivated. The amounts of this disinfectant that are currently used (1.5 mg/liter at a 3:1 ratio of chlorine to ammonia-N) may be inadequate to control the growth of these organisms in the distribution system.

Nitrification is an important microbiological process in the oxidation of ammonia in terrestrial and aquatic environments. Ammonia is converted sequentially to nitrite and nitrate by two groups of chemolithotrophic nitrifying organisms, the ammonia-oxidizing bacteria (AOB) and the nitrite-oxidizing bacteria. Nitrification has been well recognized as a beneficial treatment for the removal of ammonia in municipal sewage. In addition, research at several European water treatment facilities has shown that controlled nitrification provides an effective means of removing ammonia from the raw water (16, 26). Elimination of ammonia produces more bacteriologically stable drinking water and reduces the costs associated with additional disinfectant requirements (12). In controlled or complete nitrification, ammonia is converted to nitrate in the filter beds, where high levels of nitrifying bacteria are allowed to grow. This growth is achieved by delaying the disinfection process until after the water has passed through the filters.

In contrast to the benefits of complete nitrification, incomplete or partial nitrification in chloraminated distribution systems can adversely affect water quality. Incomplete nitrification results in the buildup of nitrite from the growth of AOB (36). Nitrite is problematic because it rapidly reduces free chlorine (26), accelerates the decomposition of chloramines (32), and can interfere with the measurement of free chlorine (18, 35). In 1939, Larson (20) reported that incomplete nitrification in a distribution system containing 1.8 mg of monochloramine residual per liter resulted in corrosion of the pipelines, increased heterotrophic plate counts (HPCs), and anaerobic conditions. Similar effects have also been reported for other systems that use chloramines, but sufficient details have not been provided to clearly determine whether the causative agents were AOB (9, 14, 21, 28). Most recently, Wolfe et al. (36) have reported nitrification episodes in two covered finished-water reservoirs in a Southern California system with a 1.5-mg/liter monochloramine residual. During the course of the episodes, the total chlorine and ammonia concentrations in the reservoirs declined rapidly, and the HPC level in one of the reservoirs exceeded 90,000 CFU/ml. Elevated levels of AOB and nitrite in the reservoirs, as compared with those in the reservoir influents, indicated that incomplete nitrification was occurring. Furthermore, the presence of AOB in the chloraminated water suggested that these bacteria were highly resistant to this disinfectant.

An understanding of how incomplete nitrification occurs in chloraminated waters is of increasing importance as many utilities convert to chloramine disinfection to comply with current and future regulations on disinfection by-products. Unfortunately, very little information is available on the occurrence or disinfection resistance of AOB in potable water. Tuovinen et al. (31) detected AOB in several tubercle

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deposits in finished-water pipelines. In their study, the presence of nitrifying bacteria was demonstrated only by measuring the pH and turbidity of cultures. No biochemical identification or enumeration of the AOB was reported. In 1935, Feben (14) reportedly isolated *Nitrosococcus* spp. from filter beds and tap water samples in a chloraminated distribution system. A number of other reports have also alluded to the presence of nitrifying bacteria in water supplies but have provided no data—or only anecdotal data—on their occurrence and identity (17, 21, 24). The lack of information on nitrifiers in water arises, in part, from methodological difficulties in isolating and enumerating these organisms. Recovery efficiencies are typically low, incubation times of several weeks or more are required, and results may be confounded by the presence of heterotrophic bacterial contaminants (4).

The purpose of this study was to examine the occurrence of AOB in a chloraminated distribution system. Emphasis was placed on monitoring AOB levels in two covered finished-water reservoirs in which previous nitrification episodes had occurred (36). Secondary objectives were to identify AOB isolated from the reservoirs, assess relationships between the numbers of AOB and selected water quality parameters, and determine the resistance of these bacteria to chloramines and free chlorine. Collectively, the findings of these studies may be helpful in developing techniques for preventing nitrification in chloraminated systems.

MATERIALS AND METHODS

System description. The Metropolitan Water District of Southern California is a public, municipal agency that wholesales supplemental water through 27 member agencies (cities and water districts), serving approximately 14 million people in a 5,200-mi^2 (13,200-km^2) service area. Water is imported from the Colorado River via the Colorado River Aqueduct and from Northern California through the California State Water Project. Water deliveries currently average approximately 2 million acre-ft (2.47×10^9 m^3) per year. The system includes five water filtration plants and four finished-water reservoirs.

Two of the reservoirs of the Metropolitan Water District, Garvey and Orange County reservoirs, have floating, synthetic rubber (Hypalon; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) covers. Garvey Reservoir and Orange County Reservoir have, respectively, capacities of 1,600 and 200 acre-ft (19.74×10^5 m^3 and 2.47×10^5 m^3), maximum depths of 51 and 46 ft (15.5 and 14.0 m), and maximum surface areas of 38 and 7.3 acres (15.4 and 3.0 ha). The water in these reservoirs was treated at the F. E. Weymouth Filtration Plant, located in La Verne, Calif. Treatment processes at all the filtration plants include coagulation, flocculation, sedimentation, filtration, and disinfection. Chlorine is added to the plant influent, and ammonia is added after the filters to obtain a 3:1 weight ratio of chlorine to ammonia-N (Cl_2/N). The chloramine residual of water entering the distribution system is approximately 1.5 mg/liter. The water leaving the plant is adjusted to approximately pH 8.2 for corrosion control. The temperature of water in the distribution system ranges seasonally from 12 to 27°C.

Typically, additional free chlorine (0.1 to 0.2 mg/liter) is applied at the effluent of the reservoirs to maintain the 1.5-mg/liter chloramine residual downstream from the reservoirs. Because of the previous nitrification episode in Garvey Reservoir, the Cl_2/N weight ratio at this reservoir is increased to 4:1 by adding chlorine at the inlet to decrease the amount of free ammonia from May to October each year. The retention time of water in Orange County Reservoir averages 4 days and ranges from 2 to 9 days, whereas the mean retention time in Garvey Reservoir is 9 days, with a range of 4 to 16 days.

Sample collection. Samples were collected from the following locations: Weymouth plant (influent and effluent), Garvey Reservoir (influent, effluent, and at a 5-ft [1.5-m] depth in the reservoir water column), and Orange County Reservoir (effluent and at a 5-ft [1.5-m] depth in the reservoir water column). With the exception of Orange County Reservoir, samples were collected every 2 weeks from January 1986 to December 1987. Orange County Reservoir samples were collected from October 1986 to December 1987. On several occasions, samples were also obtained from the interior surfaces and sediment material of two finished-water pipelines, Palos Verdes Feeder (inner diameter, 72 in [1.83 m]) and Middle Feeder (inner diameter, 50 in [1.27 m]), and from the sediment on the bottom and sides of Orange County Reservoir.

Water samples were collected in sterile bottles containing 10% sodium thiosulfate (J. T. Baker Chemical Co., Phillipsburg, N.J.) to neutralize the chloramines. Samples were transported to the laboratory on ice at 4°C and analyzed within 4 h of arrival. Sampling personnel entered the pipelines and collected samples immediately after the line was dewatered to minimize the impact of desiccation. Areas measuring 12 by 10 cm^2 were aseptically scraped at several representative sites on the Palos Verdes Feeder. For samples from the Middle Feeder, an area measuring 250 by 350 cm^2 was scraped. All scrapings were placed into sterile test tubes containing *Standard Methods* (SM) buffer (3). Also, approximately 1 ml of the sediment slurry from the bottom of the Palos Verdes Feeder was placed into 100 ml of the SM buffer. Sediment samples from the bottom and sides of Orange County Reservoir were collected by scuba divers. Approximately 4 cm^2 of the sediment material from each site was scooped with a sterile spatula into a sterile bottle containing 10% sodium thiosulfate (J. T. Baker) in SM buffer. Water samples were analyzed for the following constituents: coliforms, temperature, free and total residual chlorine, pH, nitrite-N, and free and total ammonia-N. Biofilm and sediment samples were also analyzed for dry weight, as described in *Standard Methods* (3).

Analyses. Chlorine and nitrite were measured with a colorimeter (model DR/1A; Hach Co., Loveland, Colo.). The reliability of the chlorine measurements was checked against the ferrous ammonium sulfate diethyl-*p*-phenylenediamine (FAS-DPD) procedures described in *Standard Methods* (3) with an amperometric titrator (Hach Co.). The reliability of the nitrite measurements was checked against the diazotized sulfanilic acid–*N*-(1-naphthyl)-ethylenediamine dihydrochloride method described in *Standard Methods* (3) with a continuous-flow analyzer (model AC 200; Scientific Instruments Corp., Pleasantville, N.Y.). Ammonia-N concentrations were determined with an ammonia-specific electrode (model 95-12; Orion Research, Inc., Cambridge, Mass.). Dry weight determinations of biofilm and sediment samples were made by drying the material for 30 min, or until it was completely dry, at 103°C in a convection oven (Thelco model 15; GCA/Precision Scientific Co., Chicago, Ill.) and then weighing it on an electrobalance (model 29; Cahn Instruments, Cerritos, Calif.). The HPCs were

enumerated in duplicate by both the pour plate procedure, with tryptone glucose extract agar (Difco Laboratories, Detroit, Mich.) incubated for 48 h at 35°C, and the membrane filtration technique, with R2A medium (Difco) incubated for 7 days at 28°C.

The AOB were enumerated by a five-tube most-probable-number (MPN) technique (1, 13, 36). Tenfold serial dilutions of samples were placed in 2-ml cell well plates (Corning Glass Works, Corning, N.Y.) containing medium formulated by Soriano and Walker (29). However, the medium was slightly modified by increasing the phenol red concentration to improve the sensitivity for detection of growth. The modified Soriano and Walker (MSW) medium is composed of the following ingredients per liter of distilled water: $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.04 g; $CaCl_2 \cdot 2H_2O$, 0.04 g; KH_2PO_4, 0.2 g; chelated iron (Sequestrene 138 Fe; CIBA-GEIGY Corp., Greensboro, N.C.), 0.00016 g; and phenol red, 0.002 g. The medium was adjusted to pH 8.0 with NaOH (10 N). The trays were sealed in plastic bags to prevent desiccation and incubated in the dark for 21 days at 28°C. Each cell well was tested for the presence of nitrite by adding several drops of sulfanilic acid and *N*,*N*-dimethyl-α-naphthylamine (23). Wells that exhibited a red color within 1 min after addition of the reagents were scored positive for nitrite. Wells that developed a slight pink color after 1 min or that were colorless were scored negative. Controls consisting of distilled water supplemented with MSW medium were incubated under the same conditions as the water samples. Positive controls consisted of spiking selected wells with an actively growing culture of *Nitrosomonas europaea* ATCC 19718 (American Type Culture Collection, Rockville, Md.). Because volumes of 10, 1, and 0.1 ml were being assayed, the detection limit for AOB with this system was an MPN of 0.2/ml. The AOB values presented in this report represent the mean of duplicate MPN analyses.

Isolation and identification of AOB. Nitrite-positive cultures from Orange County and Garvey reservoir samples were enriched in MSW medium. Enrichment cultures were first spread plated onto MSW medium containing 1% agar and incubated in the dark at 28°C inside plastic bags. Colonies that developed within the first 5 to 7 days were presumed to be heterotrophic bacterial contaminants, as ammonia oxidizers are relatively slow growers. Small, round, tan-colored colonies that developed after 7 days were selected for further analysis as potential nitrifiers. Colonies were picked from the agar with sterile 25-μl micropipettes (Drummond Scientific Co., Broomall, Pa.) and ejected into test tubes containing 4.0 ml of sterile MSW medium with phenol red. The tube cultures were incubated in the dark at 28°C for up to 6 weeks. Cultures that turned yellow were tested for nitrite in a spot plate. Positive tubes were transferred to Erlenmeyer flasks containing 30 ml of sterile MSW medium buffered to pH 8.0 with 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Sigma Chemical Co., St. Louis, Mo.). After turbid growth had developed, small amounts of it were plated onto agar containing MSW medium. Colonies appearing after 7 days of incubation were transferred into tubes containing MSW medium. Cultures that tested positive for nitrite were tested for the presence of heterotrophic bacterial contaminants by adding 0.5 ml of the culture to small vials containing approximately 3.5 ml of sterile R2A broth. The absence of growth in these R2A tubes after 7 days of incubation at 28°C provided presumptive evidence of pure cultures of AOB.

Actively growing cultures of AOB were identified by light and transmission electron microscopy. Pure cultures isolated from Garvey and Orange County reservoirs were grown for 2 weeks in 250-ml Erlenmeyer flasks containing MSW medium. Cells were concentrated in a centrifuge (model B20A; International Equipment Co. [IEC], Needham Heights, Mass.) at 2,500 rpm for 10 min at 4°C and suspended in sterile phosphate buffer (pH 7.2). The cells were fixed and embedded by a modification of the procedure described by Ryter and Kjellenberger (27). The bacterial suspension was fixed in 4% glutaraldehyde containing 0.1 M cacodylate buffer (pH 7.2) for 1 h at 4°C and then washed by centrifugation (model B20A, IEC) three times at 2,500 rpm for 10 min in the cacodylate buffer. The cells were fixed in 1% osmium tetroxide containing the buffer of Ryter and Kjellenberger (27) for 16 h at 23°C, washed three times by centrifugation in distilled water, and prestained in 0.5% uranyl acetate for 2 h at 23°C. The bacteria were washed twice in distilled water and suspended in a centrifuge tube with 1% warm agar. The agar-cell suspension was dehydrated with a graded series of ethanol solutions (30, 50, 70, 95, and 100%, three times each, with 10 min at each step). Excess agar was removed at the 50% ethanol step. The agar-cell suspension was dispensed in vials and rotated during infiltration with Spurr plastic embedding medium (30) at increasing gradients (30, 50, 75, and 100%, three times each, with 1.5 h of contact time at each gradient) mixed with ethanol. The vials were placed in a desiccator under house vacuum when the plastic content reached 100%. The samples were then embedded in BEEM capsules (Better Equipment for Electron Microscopy, Inc., New York, N.Y.) and cured at 60°C for 2 days. The samples were sectioned on an ultramicrotome (Ultrotome III; LKB Instruments, Inc., Stockholm, Sweden) and placed on grids (with 1-by-2-mm holes) with Formvar (Ted Pella, Inc., Redding, Calif.) and carbon support. The grids were stained with 10% uranyl acetate in methanol for 20 min and Reynolds (25) lead citrate (Ted Pella) for 5 min and studied with a transmission electron microscope (model 100C; Japan Electron Optics Laboratory, Tokyo, Japan).

Inactivation assay. A pure culture of ammonium-oxidizing bacteria, obtained through enrichment of cultures from Garvey Reservoir, was inoculated into 500 ml of either MSW medium or filter-sterilized, chlorine-neutralized tap water supplemented with 0.5 g of $(NH_4)_2SO_4$ and incubated in the dark at 28°C for 3 to 5 weeks. The bacterial suspension was filtered through a 0.2-μm-pore-size polycarbonate membrane (Nuclepore Corp., Pleasanton, Calif.) to concentrate the cells and was then rinsed three times with 50 ml of sterile SM buffer to remove chlorine-demand compounds. The membrane was placed into a sterile 50-ml centrifuge tube containing 10 ml of sterile SM buffer and gently vortexed to remove the attached bacteria.

Inactivation studies were conducted at 23°C in oven-baked, 600-ml glass beakers that were loosely wrapped with aluminum foil to shield the cultures from direct ambient light. The washed AOB cells were seeded into 200 ml of sterile, deionized-distilled potassium phosphate-buffered water (10 mM, pH 8.0) at an MPN of approximately 10^5 to 10^7/ml before the addition of the disinfectant solution. Immediately before and after each inactivation experiment, the disinfectant solutions were measured for free and total residual chlorine by the FAS-DPD titrimetric procedure (3). The experiments were initiated by adding 200 ml of double-strength (2.0-mg/liter), preformed chloramine or free chlorine to the seeded water to achieve a final disinfectant concentration of approximately 1.0 mg/liter. All test solutions were continuously mixed at 100 rpm with a paddle

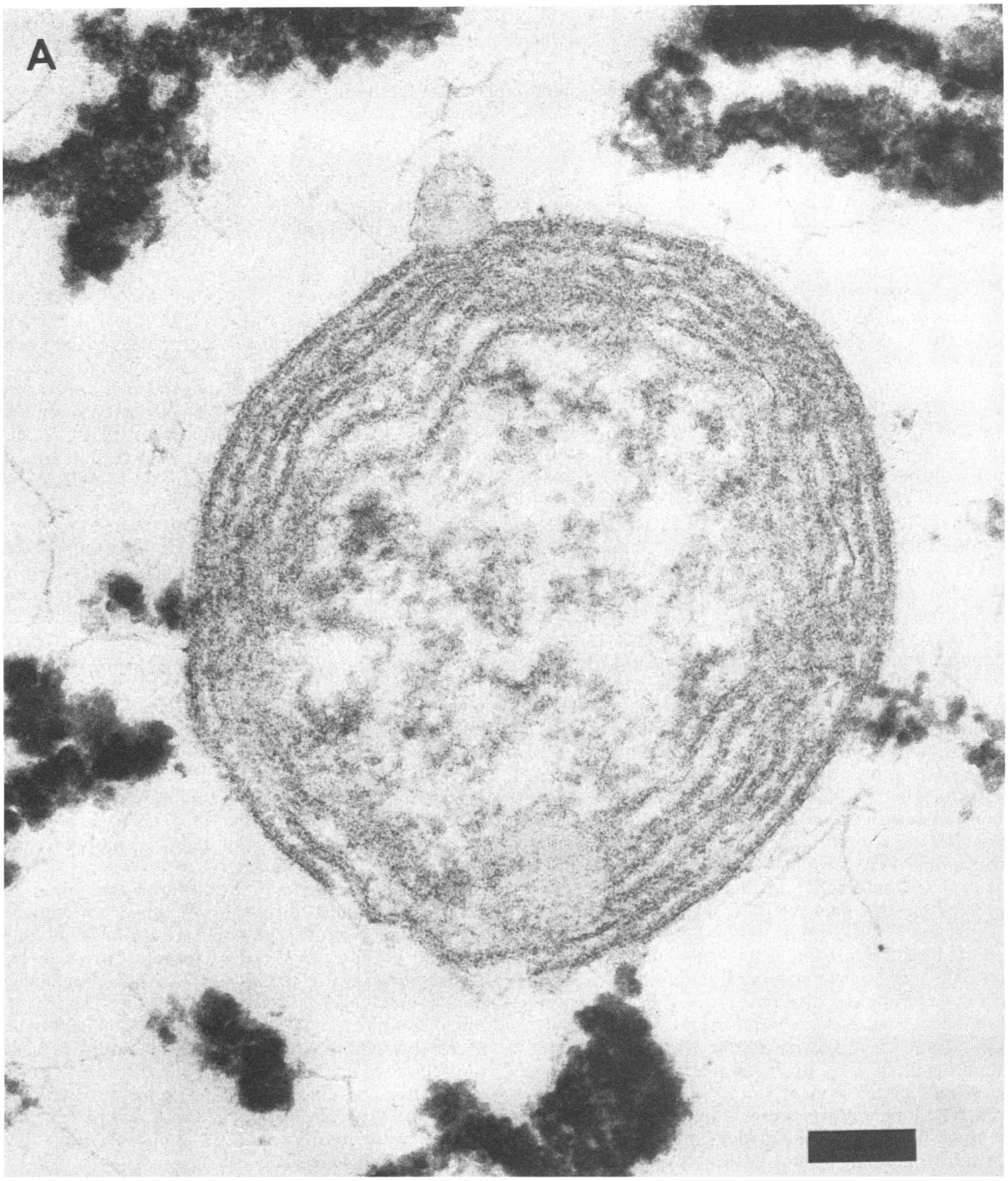


FIG. 1. (A) Transmission electron micrograph of a transverse section of an ammonia-oxidizing bacterium isolated from Garvey Reservoir. (B) Increased magnification of the electron micrograph in panel A demonstrating the presence of several layers of intracytoplasmic membranes in the peripheral region of the cell. Bars, 0.1 μm.

stirrer (Phipps & Bird., Inc., Richmond, Va.) during the assay. At preselected contact times, 10-ml portions were removed and added to sterile test tubes containing 0.10 ml of 3.0% sodium thiosulfate, vortexed, and placed into the MPN titer wells. The wells were incubated at 28°C in the dark for 21 days and then tested for the presence of nitrite.

Statistical analyses. Statistical analyses were performed with a statistical software program (RS/1, version 3.0; BBN Software Products Corp., Cambridge, Mass.) contained on a minicomputer (VAX 8200; Digital Equipment Corp., Marlboro, Mass.).

RESULTS

Identification of AOB. The AOB isolated from Garvey and Orange County reservoirs had the same morphologies and sizes. Both isolates were gram-negative, rod-shaped bacteria, 0.8 by 1.2 μm in size. Although the cells appeared

FIG. 1—*Continued*

TABLE 1. Distribution of AOB counts from monitoring sites

MPN/ml	% Distribution of AOB counts from the following locations:							
	Treatment plant		Reservoirs					
	Weymouth influent	Weymouth effluent	Garvey influent	Garvey water column	Garvey effluent	Orange County influent	Orange County water column	Orange County effluent
≤0.2[a]	9.1	48.8	37.2	39.1	27.6	66.7	27.3	18.2
0.2 to <5	59.1	41.5	62.8	41.3	51.1	33.3	45.5	48.5
5 to <20	13.6	9.8	0	10.9	17.0	0	15.2	18.2
20 to <50	9.1	0	0	6.5	4.3	0	9.1	12.1
>50	9.1	0	0	2.2	0	0	3.0	3.0
Total no. of samples	44	41	43	46	47	26	33	33

[a] Detection limit.

nonmotile in a wet-mount examination, flagellar staining revealed the presence of a polar flagellum of approximately 5 μm in length. Cells usually occurred singly or in small clumps. Transmission electron microscopy revealed the presence of extensive layers of stacked intracytoplasmic membranes in the peripheral region of the cytoplasm (Fig. 1A and B). These morphologic features are indicative of members of the genus *Nitrosomonas*.

Occurrence of AOB. AOB were detected in water samples collected from all locations over an 18-month period (Table 1), with the highest levels observed in the Weymouth plant influent and in the water column of the covered finished-water reservoirs. The majority of samples (greater than approximately 70%) at all sites contained AOB at an MPN of <5/ml. The percentages of samples containing AOB at an MPN of ≥5/ml in the plant influent and the water column sites in Garvey and Orange County reservoirs were 31.8, 19.6, and 27.3%, respectively. The lowest AOB counts were detected in the plant effluent (routinely at an MPN of <20/ml) and reservoir influent sites (routinely at an MPN of <5/ml). The concentrations of AOB in the effluents from the covered reservoirs were comparable to those in reservoir water column sites.

The seasonal occurrence of AOB in the Weymouth plant influent and effluent between December 1985 and October 1987 is depicted in Fig. 2. The numbers of AOB in the plant influent ranged from an MPN of <0.2/ml in the winter months to an MPN of >70/ml in the summer months of June, July, and August. The numbers of AOB entering the distribution system (plant effluent samples) were generally low, with MPNs ranging from <0.2 to 15/ml. On a few occasions in the fall of 1986 and the summer of 1987, the numbers of AOB were higher in the plant effluent than in the influent samples. The concentrations of AOB in the water columns and inlet sites for both reservoirs were also highest in the summer months and were typically below the detection limit in the winter months (Fig. 3 and 4). During the summer months, AOB numbers in both reservoirs were approximately 1 $\log_{10}$ unit higher than they were in the influents to the reservoirs. The highest concentration observed was at an MPN of 380/ml in Orange County Reservoir in August 1986 during a nitrification episode; the reservoir was temporarily removed from service and the water was chlorinated past the breakpoint. In both reservoirs, the numbers of AOB in the water column sites generally were higher in the summer of 1986 than in the summer of 1987.

Sediment samples collected from the bottom of Orange County Reservoir (reservoir sediment) and sediment and pipe wall scrapings collected from one of two finished-water pipelines also contained AOB. The Orange County Reservoir sediment had a floclike consistency and a dry weight of 1,100 mg/cm^2 (Table 2). The concentration of AOB in this sediment sample was at an MPN of 67/mg (dry weight). In comparison, less material was found in the finished-water pipelines. The dry weight of the pipe wall scrapings ranged from 0.0027 to 0.58 mg/cm^2, whereas the pipe sediment dry weights ranged from 0.43 to 0.56 mg/cm^2. The AOB levels were higher in the pipe sediment (MPNs, 10.4 to 12.1/mg) than in the pipe wall scrapings (MPNs, <0.2 to 7.4/mg). The pipe sediment was different from the reservoir sediment in that it had a sandlike texture. The R2A HPCs were typically 1 to 2 $\log_{10}$ units higher than the AOB levels in all sediment and pipe wall scraping samples (Table 2).

Relationship of AOB counts to selected water quality parameters. The means and ranges of selected water quality parameters are shown in Table 3. Throughout the study period, there was little variability in the concentrations of total chlorine, free and total ammonia-N, and nitrite-N compared with the variability in the temperature and levels

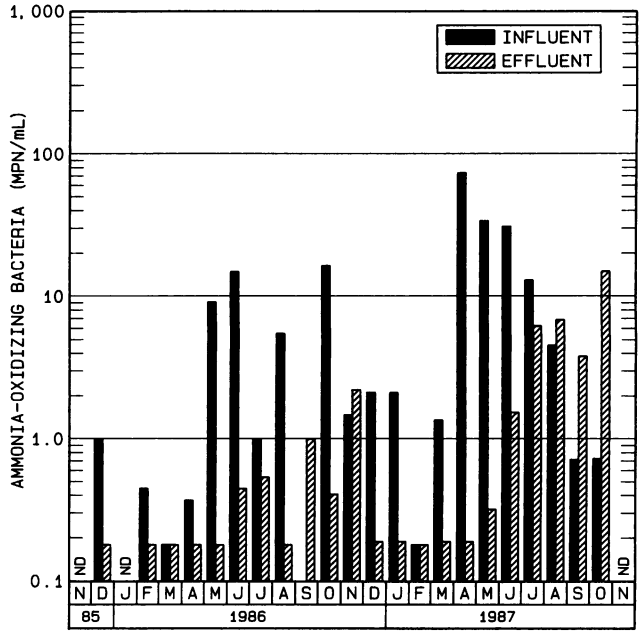


FIG. 2. Seasonal occurrence of AOB in samples collected from Weymouth plant influent and effluent. Each bar represents the geometric mean of semimonthly samples. ND, Not detected.

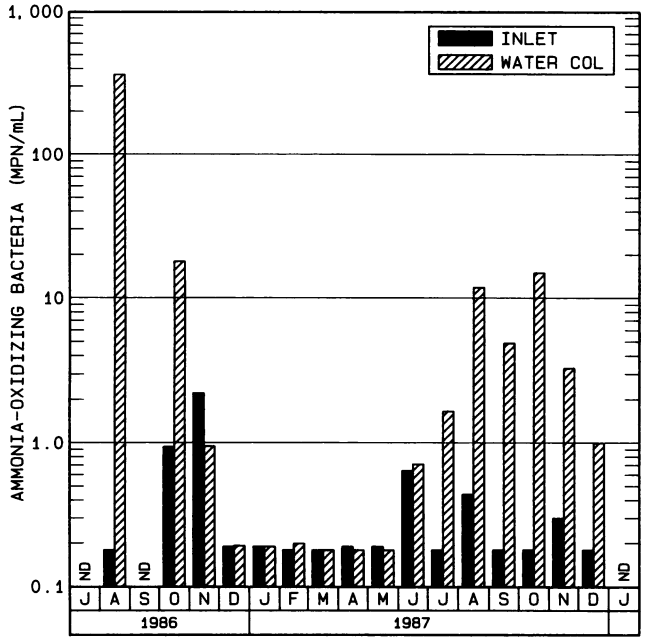


FIG. 3. Seasonal occurrence of AOB in samples collected from the water column and inlet of Orange County Reservoir. Each bar represents the geometric mean of semimonthly samples. ND, Not detected.

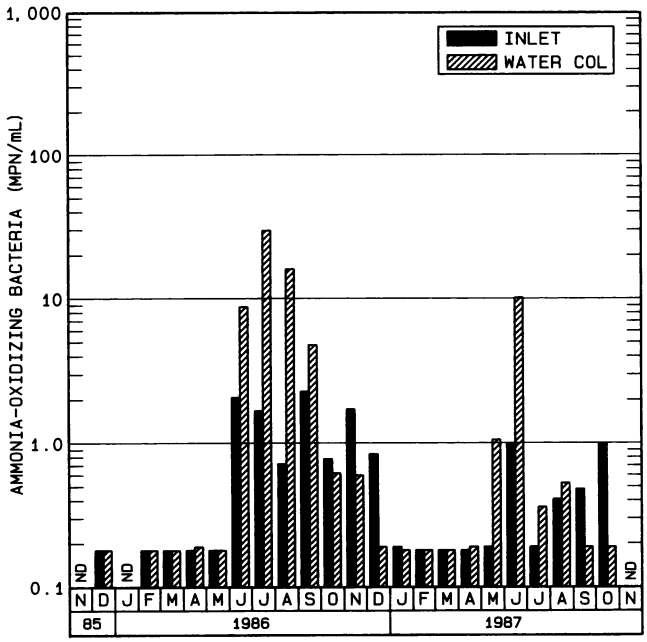


FIG. 4. Seasonal occurrence of AOB in samples collected from the water column and inlet of Garvey Reservoir. Each bar represents the geometric mean of semimonthly samples. ND, Not detected.

of HPCs. The mean total chlorine residual concentration in Orange County Reservoir (water column site) was 1.19 mg/liter, nearly 0.2 mg/liter lower than the average residual concentration in Garvey Reservoir. An average of 0.04 mg more free ammonia-N per liter was found in Orange County Reservoir than in Garvey Reservoir because of the higher Cl_2/N ratio maintained in Garvey Reservoir in the summer months. The greatest amount of nitrite, 29 μg/liter, was detected in a sample collected from Orange County Reservoir (water column site). No nitrite was detected in either the Weymouth plant influent or effluent, and less than 9 μg/liter was detected in all samples collected from Garvey Reservoir. The R2A and pour plate HPCs were highest in the Weymouth plant influent and water column sites of the reservoirs and lowest in the plant effluent and reservoir inlet sites (Table 3). In general, R2A HPCs in the reservoir sites were approximately 5 to 100 times higher than the pour plate counts. The R2A HPCs indicated a trend toward increasing HPC levels as a result of reservoir storage, whereas the pour plate HPCs either were stable or showed a decreasing trend. The geometric means for R2A HPCs in Orange County Reservoir were nearly three times higher than the counts in Garvey Reservoir (water column sites). With the exception of the Weymouth plant influent, none of the sites had pour plate counts with a geometric mean of >50 CFU/ml.

Coefficients of correlation between the levels of AOB at the various sites and selected water quality characteristics are shown in Table 4. Statistically significant positive correlations between the concentrations of AOB and water temperature were observed at all sites except the Weymouth plant influent and the Orange County Reservoir inlet. The highest temperature-AOB correlations were observed for

TABLE 2. Analyses of pipe wall and sediment samples from finished-water mains and Orange County Reservoir

Location	Sample date (mo/day/yr)	Sample	Dry wt/area (mg/cm²)	AOB (MPN)		R2A HPCs (CFU)	
				Cells/cm²	Cells/mg	Cells/cm²	Cells/mg
Palos Verder Feeder	2/4/87	Pipe wall scraping	0.110	0.8	7.4	0.75	6.70
	2/4/87	Pipe wall scraping	0.510	<0.2	<0.2	10.9	21.2
	2/4/87	Pipe wall scraping	0.580	<0.2	<0.2	15.0	25.9
	2/4/87	Pipe sediment	0.560[a]	61.2[a]	12.1	2.9×10^{3}[a]	5.2×10^{3}
	2/4/87	Pipe sediment	0.430[a]	40.5[a]	10.4	1.9×10^{3}[a]	4.4×10^{3}
Middle Feeder	2/26/87	Pipe wall scraping	0.0027	<0.2	<0.2	0.006	2.4
	2/26/87	Pipe wall scraping	0.0078	<0.2	<0.2	0.02	2.9
Orange County Reservoir	8/5/87	Side sediment	ND[b]	2.0×10^{5}	ND	5.5×10^{5}	ND
	8/5/87	Bottom sediment	ND	4.2×10^{5}	ND	3.8×10^{6}	ND
	9/3/87	Bottom sediment	1,100	3.9×10^{4}	67	7.0×10^{5}	1.4×10^{3}

[a] Results are based on a 1-ml volume of sediment and are not per square centimeter.
[b] ND, Not determined.

TABLE 3. Concentrations of selected water quality parameters at sample locations

Location	Measure	Temp (°C)[a]	Pour plate HPC (CFU/ml)[b]	R2A HPC (CFU/ml)[b]	Nitrite-N (μg/liter)[a]	Total ammonia (mg/liter)[a]	Free ammonia (mg/liter)[a]	Total chlorine (mg/liter)[a]
Weymouth plant influent	Mean	18.6	300	2,810	<5	0.015	0.041	ND[c]
	Range	12.0–25.5	6–61,000	97–35,000	<5–5	0.01–0.03	0.00–0.24	
	n[d]	44	43	39	40	39	8	
Weymouth plant effluent	Mean	18.5	3	9	<5	0.49	0.25	1.51
	Range	12.0–27.0	1.0–6,350	1.0–900	<5–5	0.41–0.55	0.18–0.30	1.4–1.7
	n	41	48	44	40	43	42	43
Garvey Reservoir influent	Mean	18.8	3	20	<5	0.49	0.27	1.39
	Range	12.0–25.0	1.0–800	1.0–1,900	<5–6	0.40–0.61	0.19–0.35	1.1–1.6
	n	48	30	46	49	49	43	49
Garvey Reservoir water column[e]	Mean	19.0	8	99	<5	0.47	0.24	1.37
	Range	12.5–26.0	1.0–9,400	2.0–5,200	<5–9	0.40–0.56	0.00–0.38	0.80–1.6
	n	50	46	47	48	49	45	47
Garvey Reservoir effluent	Mean	19.6	4	89	<5	0.46	0.23	1.46
	Range	12.5–27.5	1.0–200	1.0–2,280	<5–9	0.23–0.55	0.05–0.31	1.3–1.6
	n	49	47	47	48	49	44	49
Orange County Reservoir influent	Mean	22.2	10	52	<5	0.49	0.28	1.40
	Range	14.0–25.5	1.0–1,240	1.0–2,080	<5–11	0.36–0.57	0.17–0.42	0.80–1.6
	n	33	30	32	29	29	18	30
Orange County Reservoir water column[e]	Mean	20.8	4	280	10	0.46	0.27	1.19
	Range	13.0–25.0	1.0–770	4.0–21,000	<5–29	0.39–0.51	0.19–0.32	0.75–1.5
	n	45	36	36	38	37	26	40
Orange County Reservoir effluent	Mean	20.6	2	240	8	0.46	0.23	1.48
	Range	12.5–25.0	1.0–320	9.0–3,300	<5–21	0.38–0.51	0.17–0.28	1.4–1.6
	n	39	34	34	37	36	27	37

[a] Geometric mean.
[b] Arithmetic mean.
[c] ND, Not determined.
[d] *n*, Number of samples.
[e] Samples collected from a 5-ft (1.5-m) depth in the reservoir water column.

samples collected from the water column (r = 0.84) and effluent sites (r = 0.84) of Orange County Reservoir. The R2A HPCs correlated more with the AOB levels than did the pour plate HPCs at all sites except the Weymouth plant influent and effluent and the Garvey Reservoir effluent (Table 4). The highest HPC-AOB correlations were observed in the Orange County Reservoir water column (r = 0.78) and effluent (r = 0.65) sites. With the exception of the Orange County Reservoir site, few statistically significant correlations between the levels of AOB and nitrite-N, free and total ammonia, and total chlorine were found (Table 4).

Temperature, R2A HPCs, and nitrite-N were the factors that were most highly correlated with AOB in the Orange County Reservoir water column site (Fig. 5A to C). No AOB were detected in the reservoir when the water temperature was less than 18°C. Above this value, the numbers of AOB generally appeared to increase in relation to temperature (Fig. 5A). Few AOB were observed when the numbers of

TABLE 4. Pearson r coefficients of correlation between AOB concentrations and selected water quality parameters

Location	r coefficients of correlation for the following parameters[a]:						
	Temp (°C)	Pour plate HPC	R2A HPC	Nitrite-N	Free ammonia	Total ammonia	Total chlorine
Weymouth plant influent	0.11	0.04	0.35[b]	ND[c]	ND	ND	ND
Weymouth plant effluent	0.60[b]	0.41[b]	0.32[b]	ND	0.06	0.04	−0.30
Garvey Reservoir inlet	0.54[b]	0.13	0.46[b]	−0.02	0.07	−0.01	−0.41[b]
Garvey Reservoir	0.58[b]	0.31	0.50[b]	0.28	−0.01	−0.18	−0.31
Garvey Reservoir effluent	0.63[b]	0.62[b]	0.46[b]	0.18	0.06	−0.12	−0.31
Orange County Reservoir inlet	0.14	0.15	0.08	0.01	−0.21	0.11	0.01
Orange County Reservoir	0.84[b]	0.08	0.78[b]	0.74[b]	0.43	0.56[b]	−0.83[b]
Orange County Reservoir effluent	0.84[b]	−0.03	0.65[b]	0.57[b]	−0.59[b]	−0.15	0.32

[a] Correlations were based on $\log_{10}$ values for AOB, pour plate HPC, and R2A HPC data.
[b] Statistically significant correlations at α = 0.05.
[c] ND, Not determined.
[d] Samples collected from a 5-ft (1.5-m) depth in the reservoir water column.

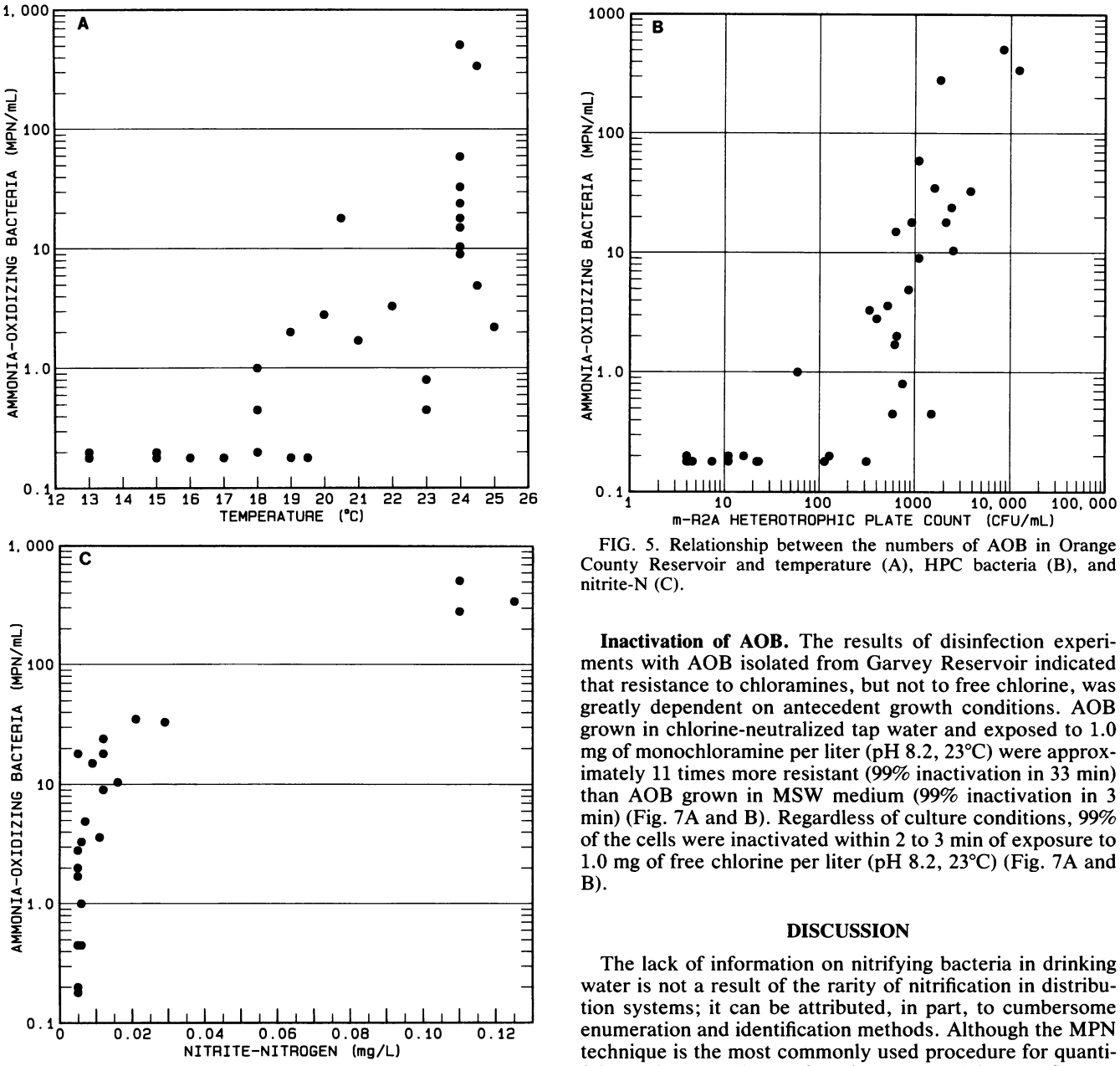


FIG. 5. Relationship between the numbers of AOB in Orange County Reservoir and temperature (A), HPC bacteria (B), and nitrite-N (C).

R2A HPCs were less than approximately 350 CFU/ml. Above this level, the number of AOB also increased linearly in relation to HPC increments (Fig. 5B). In general, the levels of R2A HPCs in Orange County Reservoir were 100 times higher than the AOB levels. A threshold relationship existed between AOB and nitrite-N levels in Orange County Reservoir samples (water column site), whereby nitrite levels generally remained below 0.01 mg/liter until the detected numbers of AOB exceeded an MPN of approximately 10/ml (Fig. 5C).

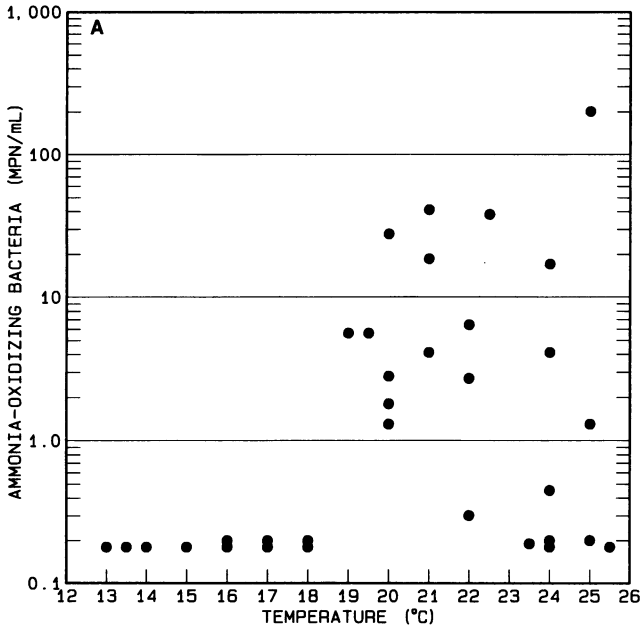
Similar threshold relationships between AOB and temperature and between AOB and HPCs were also observed in Garvey Reservoir (Fig. 6A and B). However, no relationship appeared to exist between AOB and nitrite in Garvey Reservoir, as AOB concentrations ranged from MPNs of <0.2 to 200/ml at nitrite concentrations of 0.005 mg/liter (Fig. 6C).

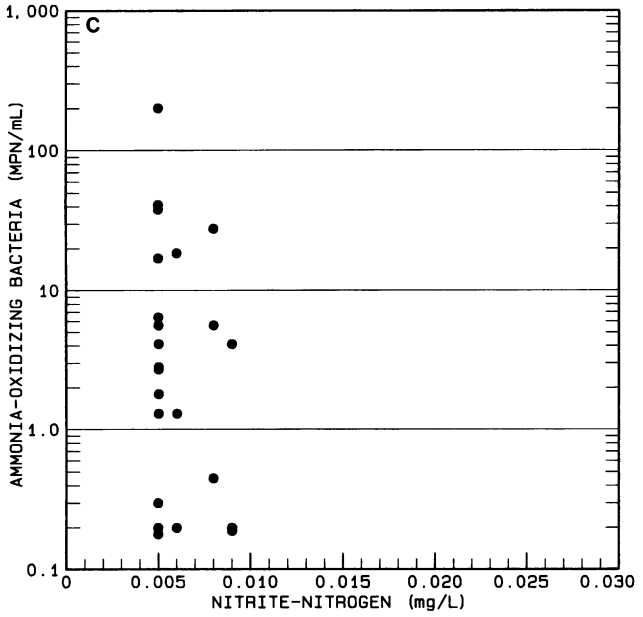
Inactivation of AOB. The results of disinfection experiments with AOB isolated from Garvey Reservoir indicated that resistance to chloramines, but not to free chlorine, was greatly dependent on antecedent growth conditions. AOB grown in chlorine-neutralized tap water and exposed to 1.0 mg of monochloramine per liter (pH 8.2, 23°C) were approximately 11 times more resistant (99% inactivation in 33 min) than AOB grown in MSW medium (99% inactivation in 3 min) (Fig. 7A and B). Regardless of culture conditions, 99% of the cells were inactivated within 2 to 3 min of exposure to 1.0 mg of free chlorine per liter (pH 8.2, 23°C) (Fig. 7A and B).

DISCUSSION

The lack of information on nitrifying bacteria in drinking water is not a result of the rarity of nitrification in distribution systems; it can be attributed, in part, to cumbersome enumeration and identification methods. Although the MPN technique is the most commonly used procedure for quantifying AOB, membrane filtration (15) and immunofluorescence techniques have been used on a limited basis (6). The MPN technique requires varied incubation times, ranging from 20 to 55 days, depending on the recovery medium that is used and the temperature of incubation. In addition, the bacteria must be incubated in the dark, as sunlight—or even ambient fluorescent light—inhibits the growth of these bacteria (2). This study used MSW medium, with incubation for 3 weeks at 28°C, as these conditions have been found to produce the most rapid growth of AOB (4, 22). The recovery efficiency of this technique has been reported to range from 0.1 to 5% (5). Therefore, concentrations of AOB presented in this and other studies that use the MPN procedure should be evaluated on a relative rather than an absolute scale.

Biochemical and microscopic analyses of cultures isolated from the reservoirs indicated that these bacteria had morphologic features characteristic of the genus *Nitrosomonas*. Members of this genus typically have ellipsoidal or rod-





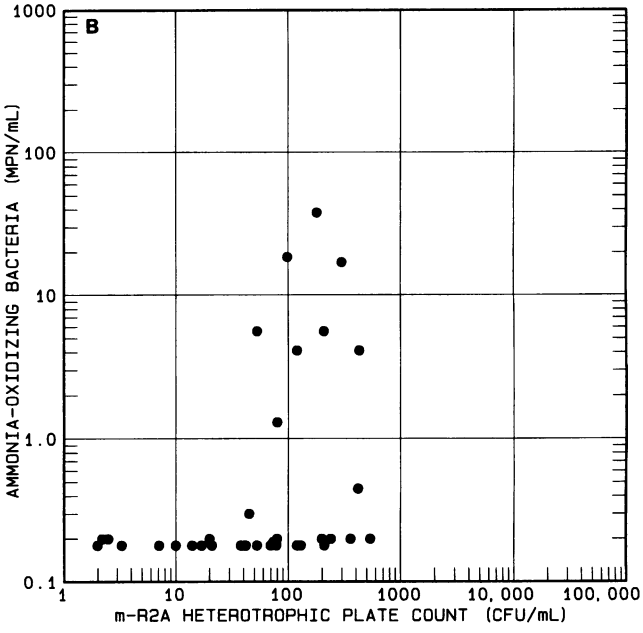


FIG. 6. Relationship between the numbers of AOB in Garvey Reservoir and temperature (A), HPC bacteria (B), and nitrite-N (C).

shaped cells with rounded or pointed ends. Most strains are motile, with polar flagella, and possess stacked intracytoplasmic membranes in the peripheral region of the cytoplasm (8, 34). *Nitrosomonas* spp. are commonly found in soil and aquatic environments. However, the media and incubation conditions used in this study may have been selective for the recovery of *Nitrosomonas* spp. and no other AOB (7).

Results of the monitoring program indicated that although conventional water treatment processes generally reduced the numbers of AOB, some organisms were able to survive and enter the distribution system. On occasion, the numbers in the filter effluent were higher than those in the source water, suggesting that favorable growth conditions periodically existed in the treatment plant. The numbers of AOB in the reservoirs were generally much higher in the water column than in the influent and approximately 100 to 1,000 times higher in the summer than in the winter months, indicating that these organisms were not only able to survive in the presence of 1.2 to 1.5 mg of monochloramine residual per liter but were also capable of growing in the presence of these disinfectant levels.

The elevated levels of AOB in the reservoirs undoubtedly resulted from favorable growth conditions in this environment. These bacteria grow best under conditions of mild alkalinity (pH values of 7.5 to 8.5), warm water temperatures (25 to 28°C), darkness, extended detention times, and the presence of free ammonia (34). At times, all of these conditions occur in Garvey and Orange County reservoirs. The results of this study suggested that the most significant factor for the proliferation of AOB was temperature. AOB were detected only when the water temperature was above 16 to 18°C. Similar temperature thresholds have been observed for other aquatic bacteria, including *Legionella* spp. (11). Another important factor that may have contributed to the survival of AOB in the distribution system and reservoirs was their presence in the sediment and biofilm material. These environments typically contain higher numbers of AOB, presumably because of the larger amounts of essential nutrients there (13), and provide protection from disinfection.

Although it was not examined in this study, another factor contributing to the proliferation of AOB in the reservoirs is retention time (36). Because AOB grow relatively slowly, an increase in the reservoir retention time would allow the organisms to proliferate, especially in the warm summer months. However, it is difficult to quantify the effect of detention time on the growth of AOB in a distribution system because detention time fluctuates dramatically in relation to downstream water demands and operational conditions. Moreover, reservoir volume and circulation efficiency may also influence AOB growth patterns.

In general, AOB concentrations correlated poorly with levels of nitrite, ammonia, and chlorine at all locations except Orange County Reservoir. This was presumably because of the small amount of variability in the concentra-

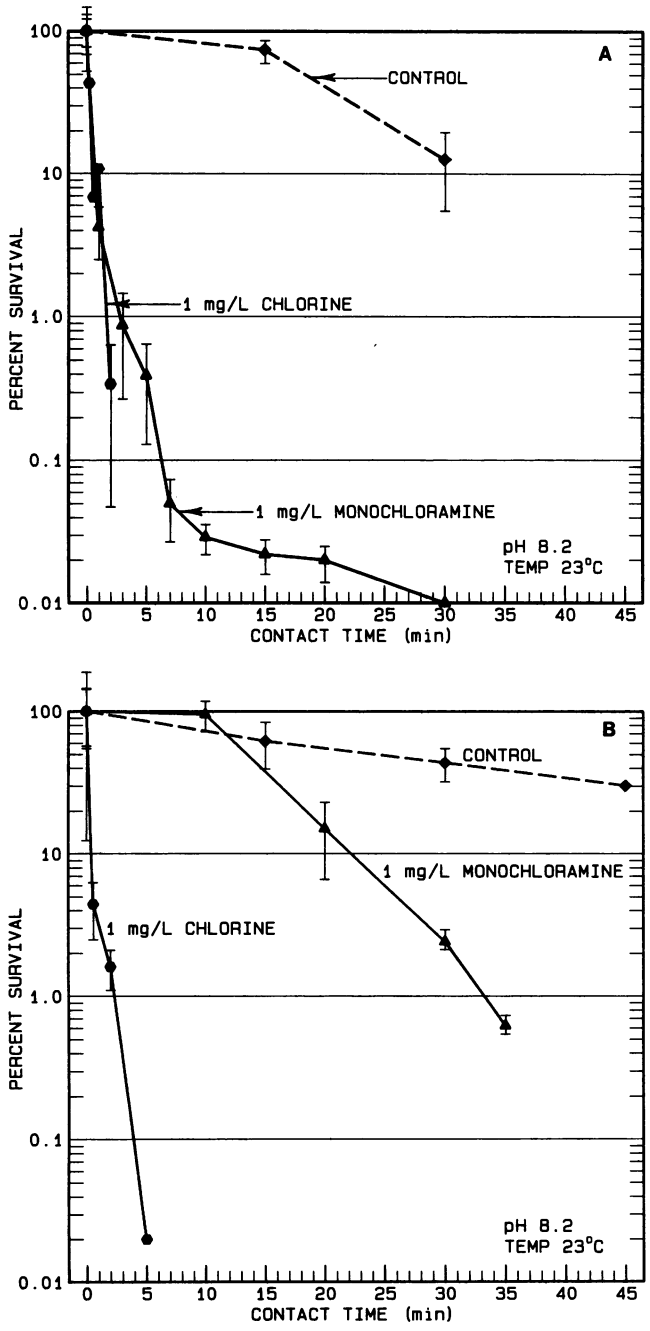


FIG. 7. Inactivation of a strain of AOB isolated from Garvey Reservoir. Cells were cultured in MSW medium (A) and tap water supplemented with ammonium sulfate (B); they were then exposed to 1.0 mg of monochloramine or free chlorine per liter at 23°C and pH 8.2.

tions of these compounds throughout the sampling period. The correlations are likely to be higher during severe nitrification episodes, when there is a wide range of nitrite and AOB levels. At Garvey Reservoir, AOB were recovered even when the concentration of nitrite was below detectable levels. This suggests that the measurement for AOB is a more sensitive indicator of nitrification than nitrite analysis and that the nitrite had already been converted to nitrate. The AOB levels correlated highly with the R2A HPCs in one of the reservoirs, suggesting that these heterotrophs may be good indicators of nitrifiers in some chloraminated systems. A surrogate measurement would be useful because of the lengthy incubations for enumerating these bacteria.

The results of disinfection experiments with AOB isolated from Garvey Reservoir indicated that these bacteria were considerably more resistant to chloramines than to free chlorine, but this was dependent on antecedent growth conditions. Cells that were grown in the chlorine-neutralized tap water were approximately 11 times more resistant to monochloramine than were cells grown in MSW medium. This increase in the disinfection resistance of bacteria grown in tap water has been shown for a number of bacteria (10, 19, 37) and may be a result of biochemical changes in the cellular envelope that prevent penetration by the disinfectant. The resistance of these bacteria to chloramines was not appreciably greater than that reported for a number of other bacteria, including *Escherichia coli* (33, 38). Consequently, the levels of chloramines typically used for potable water disinfection (1.0 to 2.0 mg/liter) should be sufficient to eliminate these organisms. However, the fact that AOB were found to proliferate in the chloraminated reservoirs suggests that the experimental protocol used to evaluate the disinfection resistance of AOB in our study may not have accurately reflected disinfection in the reservoirs.

Also, the AOB cells were well mixed prior to exposure to the disinfectants in our study. This exposure condition may not have accounted for in situ resistance mechanisms resulting from the association of the bacteria within the sediment matrix of the reservoirs and pipelines. Additional studies investigating the nature and sources of the sediment material and its role in providing resistance to disinfection are needed to determine effective chloramine concentrations. In contrast to the results described here, earlier work by Feben (14) showed that a strain of AOB isolated from tap water and grown on synthetic medium was 60 times more resistant than *E. coli* to free chlorine. However, the Feben study (14) did not measure the final chlorine residuals or wash the cells to remove nitrite and other chlorine demand compounds.

Another measure for controlling the growth of AOB would be to reduce the amount of free ammonia in chloraminated waters. This could be achieved by increasing the Cl_2/N ratio. The Metropolitan Water District of Southern California maintains a 3:1 ratio in the distribution system. At a 1.5-mg/liter total chlorine concentration, this ratio results in approximately 0.2 mg of free ammonia per liter. The free ammonia could be completely eliminated by increasing the ratio to 5:1. The higher Cl_2/N ratio maintained in Garvey Reservoir during the summer of 1987 may have accounted for the fact that the AOB level in the reservoir was lower in 1987 than in 1986. Further experiments in which the Cl_2/N ratio is adjusted should be performed to determine the effects of free ammonia on the growth of AOB.

In summary, the results of this study indicate that AOB occur throughout the chloraminated distribution system, with the highest levels detected in the covered reservoirs during the warm summer months. The persistence and growth of these bacteria in the reservoirs may result from their association with sediment material in the reservoirs and biofilms in the distribution pipelines. These results suggest that chloraminated systems with nitrification problems may have to increase their residuals substantially above 1.5 mg/liter to control nitrifier growth. Alternatively, utilities may be able to control the development of AOB populations by increasing the Cl_2/N ratio to reduce the amount of free ammonia-N. Temperature appears to be an important vari-

able for the growth of AOB in the reservoirs. The study also suggests that HPCs can be useful as indicators of the presence of AOB. The current enumeration techniques for AOB are lengthy and cumbersome and have a low recovery efficiency; an improved enumeration method is needed.

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