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Detection and Analysis of Two Serotypes of Ammonia-Oxidizing Bacteria in Sewage Plants by Flow Cytometry

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Two different serotypes of the genus *Nitrosomonas* were isolated from samples of the sewage plant Heidelberg. These nitrifiers were enumerated in activated sludge of various other sewage plants after immunofluorescent labeling and staining with propidium iodide by flow cytometry. The concentrations of these serotypes of *Nitrosomonas* spp. were in the range of 0.1 to 2%. Also, a test for the determination of the activity of ammonia-oxidizing bacteria was developed. Nitrite-oxidizing bacteria were specifically inhibited with sodium chlorate, and the activity of ammonia-oxidizing bacteria could be calculated from the increase of nitrite. Concentrations and activities of ammonia oxidizers were measured for a period of 6 months in the sewage plant Heidelberg. With one exception, activities and concentrations of ammonia-oxidizing bacteria decreased and increased in parallel.

In recent years, increasing emphasis has been laid on reducing the ammonia content of wastewater by microbial nitrification. This process is performed by chemoautotrophic nitrifiers in two steps. The first step, the oxidation of ammonia, is catalyzed by ammonia-oxidizing bacteria, and in the second step the resulting nitrite is oxidized by nitrite-oxidizing bacteria to nitrate. Nitrifiers grow slowly and are known to be highly sensitive to toxic substances, pH, and temperature. Thus, many sewage plants have great difficulties maintaining a stable nitrification throughout the year.

In a sewage plant, nitrification is affected by the number and specific activity of nitrifiers. Rapid evaluation of these parameters has not been possible, since fast methods for the enumeration of these bacteria are not available. The total activity of the nitrifying biomass is measured by either incorporation of [^{14}C]bicarbonate (7) or rate of ammonia oxidation (14). The concentration of these bacteria can be determined by plating onto selective media (13) or by the most-probable-number (MPN) technique (21), both of which require long incubation times of up to 8 weeks with a poor and inconsistent counting efficiency.

Furthermore, nitrifiers can be detected in samples by immunofluorescence under the fluorescence microscope (12), a fast method used for the determination of serotypes in MPN tubes (6) and for direct quantification of nitrifiers in marine environments (30, 31) and stabilization ponds (1), but which failed in biofilms (28).

In contrast to fluorescence microscopy, flow cytometry (FCM) allows the analysis of up to 1,000 bacteria per s, and several fluorescent and light-scattering characteristics of a single cell can be analyzed simultaneously. The method has been used mainly for the analysis of eucaryotic cells (19) and also is important in the analysis of bacteria in pure cultures (22, 24, 27) and environmental or clinical specimens (11, 16, 20, 25).

In this study, the concentrations of two different serotypes of *Nitrosomonas* spp. were determined in activated sludge from domestic and industrial sewage plants by this technique. The results were compared with the activity of ammonia-oxidizing biomass, measured in vitro by accumulation of nitrite. Both *Nitrosomonas* serotypes were labeled by immunofluorescence, and total bacteria in the sludge were stained with a fluorescent dye for DNA, propidium iodide. By dual-parameter analysis of both immuno- and DNA fluorescence, it was possible to detect and enumerate *Nitrosomonas* spp. among the other bacteria in activated sludge.

MATERIALS AND METHODS

Isolation of nitrifying bacteria and MPN techniques. Samples were taken from the municipal sewage plant Heidelberg. The organic loading of this plant varies between 0.25 (ca. 0.113 kg) and 0.35 lb of 5-day biochemical oxygen demand per lb of mixed-liquor suspended solids, and the sludge age varies between 4 and 10 days. The temperature is, depending on the season, in the range of 10 to 20°C, and the nitrification rate (percentage of ammonia removed) is between 10 and 80%, depending on the temperature of the incoming wastewater and other factors.

Activated-sludge samples were dispersed in a homogenizer shortly after collection (Potter-Elvehjem homogenizer; Braun, Melsungen, Federal Republic of German [F.R.G.]) and serially diluted with phosphate-buffered saline (PBS), pH 7.2. The medium for the MPN determination was as described by Soriano and Walker (26). Each MPN tube contained 5 ml of medium and was inoculated with 1 ml of the serial dilution. For each serial dilution, four tubes were prepared and incubated for 8 weeks at 30°C. A MPN tube was considered positive when >2 mg of nitrite or nitrate per ml was detected. To obtain pure cultures, bacteria from positive MPN tubes were counted under the microscope and diluted in MPN medium to a final concentration of 0.5 cell per ml; 48 tubes, each containing 5 ml of medium, were inoculated with 1 ml of this dilution. Reincubation was done for 8 weeks under the conditions given above. This procedure was repeated until pure cultures were obtained. A culture was judged pure when all cells looked uniform under a light microscope (×1,000 magnification) and no growth was

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observed within 2 weeks in thioglycolate and casein soy peptone media (Merck, Darmstadt, F.R.G.).

Antisera. To obtain antisera, 8-week-old female New Zealand White rabbits were immunized intravenously five times at 3-day intervals with 1 ml of PBS containing increasing numbers of bacteria (0.1×10^8, 0.2×10^8, 0.4×10^8, 0.8×10^8, and 1.6×10^8 cells per ml). After a 10-day rest, a boost injection was given, and 10 days later blood (20 ml) was drawn from the marginal ear vein. The day before the immunization schedule started, 20 ml of blood was taken for control serum. The sera were stored at −20°C and diluted in PBS with 2% bovine serum albumin before use.

Fluorescence microscopy. Immunofluorescence studies were performed as described by Bohlool and Schmidt (8). A film of 4% hydrolyzed gelatine was dried on top of the bacteria, fixed in 70% ethanol on glass slides. Diluted antisera were added, and after incubation, washing, and drying, samples were reincubated with fluorescein isothiocyanate-labeled secondary antibodies (swine anti-rabbit immunoglobulin antiserum; Dakopatts, Denmark), diluted 1:100 in PBS–2% bovine serum albumin. After washing the slides with PBS and drying, the slides were covered with alkaline glycerol (9 parts of glycerol with 1 part of 0.5 M Na_2CO_3-$NaHCO_3$, pH 9.0) and examined under the fluorescence microscope.

Sample preparation for FCM analysis. Activated sludge was homogenized as described above and centrifuged for 30 s at $30 \times g$ to remove crude particles. The supernatant was decanted, washed two times (10 min, $1{,}000 \times g$) with 10 mM Tris hydrochloride (2-amino-2-hydroxymethyl-1,3-propandiol) (pH 7.5), and fixed in 70% ethanol. These samples can be stored for several weeks. Prior to the staining procedure, the samples were pelleted by centrifugation, suspended in a mixture of 20 parts of 70% ethanol and 1 part of a saturated solution of barium hydroxide (Serva, Heidelberg, F.R.G.), and incubated for 1 h at room temperature to hydrolyze the RNA (9). After two washes with Tris hydrochloride, the cells were suspended in PBS–2% bovine serum albumin to a density of approximately 10^8 cells per ml and allowed to incubate for 1 h. The following incubations were performed in 96-well microdilution plates (Millititer system; Millipore Corp., Bedford, Mass.) sealed underneath with a porous polyvinyldifluoride membrane (pore size, 0.2 μm). For washing procedures, the plates were placed on a filter manifold (Millipore), which was attached to an aspirator pump.

Per well, 100 μl of the bacterial suspension and 100 μl of diluted antiserum were incubated at room temperature for 30 min. After several washes with PBS containing 0.05% Tween 20, the bacteria were incubated with 200 μl of fluorescein isothiocyanate-conjugated secondary antibodies (see above) for 30 min. After washing with PBS–0.05% Tween 20, the bacteria were suspended in PBS with 1 μg of propidium iodide (Serva) per ml. Prior to analysis by FCM, the bacteria were dispersed by suction through a hypodermic needle (interior diameter, 0.5 mm). All solutions and sera were passed through sterile filters before use.

FCM analysis. The bacteria were analyzed on an Ortho 50H Cytofluorograph (Ortho Diagnostic System, Inc., Westwood, Mass.), equipped with an argon ion laser (Coherent Innova 90; Coherent, Inc., Palo Alto, Calif.) and operated at 40 mW. The 488-nm line was used to excite the fluorescence of propidium iodide and fluorescein isothiocyanate, and the data were analyzed with an Ortho 2150 data analysis system. Prior to analysis, the instrument was calibrated with 0.57-μm microspheres (Fluoresbrite beads of 0.57-μm diameter; Polysciences Inc., Warrington, Pa.). Membrane-filtered PBS was used as the sheath fluid.

Determination of activity of ammonia-oxidizing bacteria. Activated sludge was mixed with an equal volume of MPN medium, and sodium chlorate, a selective inhibitor of nitrite oxidizers (3, 15), was added to a final concentration of 20 mM. The samples were incubated at 30°C and aerated by an aquarium pump. Samples were withdrawn at various times, and the concentration of nitrite in the supernatant was determined after centrifugation. Activities were calculated from the linear increase of nitrite during the initial 15 min of incubation. Details of this test are described elsewhere (29a).

RESULTS

Isolation of ammonia-oxidizing bacteria. Ten strains of autotrophic ammonia oxidizers were isolated from activated sludge of the sewage plant Heidelberg. All were rod shaped and so were denoted as *Nitrosomonas* spp., and antisera were raised against four of those strains. By comparing cross-reactivities, it was possible to assign all isolates to the two serotypes A10 and A13. Sera could be diluted 300-fold for immunofluorescence and showed no cross-reactivity with the other serotype and 20 heterotrophic isolates of the sewage plant even at a 20-fold dilution. Further, no cross-reactivity was obtained with a strain of *Nitrosomonas europaea*, ATCC 25928.

With these antisera, we tried to detect and quantify *Nitrosomonas* spp. in activated sludge by fluorescence microscopy. However, immunofluorescent bacteria were even observed with control sera, and an intense green background fluorescence complicated a visual identification of *Nitrosomonas* spp. For detection of bacteria in soil by immunofluorescence, this background fluorescence was suppressed by a layer of hydrolyzed gelatin applied on top of the sample before incubation with the antibodies (8). Samples of activated sludge were only slightly improved by this technique.

Analysis of ammonia-oxidizing bacteria by FCM. Figure 1 shows a dual-parameter histogram from an FCM analysis of a pure culture of *Nitrosomonas* serotype A13 after labeling with the homologous antiserum. All bacteria were red fluorescently stained by the DNA dye propidium iodide and 90% were green fluorescent, due to labeling with antibodies.

In Fig. 2a, bacteria from activated sludge were immunofluorescently labeled with antiserum against *Nitrosomonas* serotype A13 and stained with propidium iodide. A small population of green and red fluorescent bacteria, whose intensity is above channel 60 of green fluorescence, can be seen, and it accounts for 0.5% of the total bacteria in activated sludge (red fluorescent particles with an intensity above channel 20). Some 0.1% of all signals are visible in the same area in the dual-parameter histogram of an analysis of the same sample after staining with the control serum of the same rabbit before immunization with *Nitrosomonas* serotype A13 (Fig. 2b). These signals do not form a distinct population and are probably caused by bacteria which were labeled by nonspecific absorption of antibodies or binding of specific antibodies already present in the rabbit before immunization. For quantification of *Nitrosomonas* spp. in activated sludge, concentrations of bacteria labeled "immunofluorescent" with control serum (Fig. 2b) were therefore substracted from those labeled with antiserum (Fig. 2a). Thus, the activated sludge analyzed for Fig. 2a and b

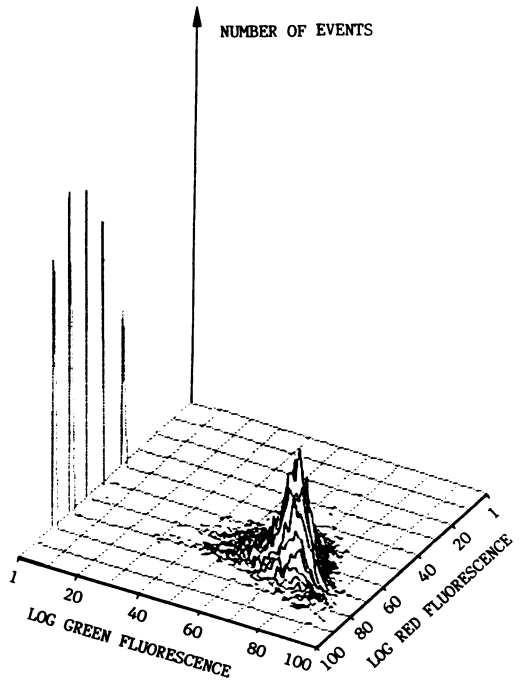

FIG. 1. Typical cytofluorographic profile of a pure culture of *Nitrosomonas* serotype A13, labeled with the homologous antiserum. Green fluorescence represents labeling with antiserum and red fluorescence represents staining of all bacteria in the sample with the DNA dye propidium iodide. The red and green fluorescence parameters were measured after three-decade logarithmic amplification and divided into 100 channels. The number of events, represented on the *z* axis, is on a linear scale. The units of the axes are arbitrary but constant for this and all subsequent figures. All bacteria were stained red by the DNA dye propidium iodide, and 90% were stained green by fluorescein isothiocyanate-conjugated antiserum. The signals of the green labeled bacteria (90%) form a cluster above channel 60 of green fluorescence, whereas the signals of bacteria that have not reacted with the antiserum (10%) are shown on the base line of green fluorescence.

contained *Nitrosomonas* serotype A13 in a concentration of 0.4% of the total bacteria, which were detected by their red DNA fluorescence.

Bacteria from pure cultures of *Nitrosomonas* serotype A13 were added to the same activated sludge prior to homogenization, fixation, and labeling with antibodies to determine their recovery rate. The concentration of total bacteria per volume of activated sludge was determined by FCM after homogenization, fixation, and staining with propidium iodide without prior centrifugation. Fluorescent microspheres were added to a final concentration of 10^7/ml, and the concentration of bacteria was calculated from the ratio of microspheres to red fluorescent signals. A concentration of 5 $\times$ 10^8 bacteria per ml was determined and *Nitrosomonas* serotype A13 was added in a ratio of 1:40. In the dual-parameter histogram of the FCM analysis (Fig. 3), a distinct population of immunofluorescent bacteria can be seen in the same area where *Nitrosomonas* spp. from pure cultures were measured (Fig. 1). These bacteria account for 2.9% of all red fluorescent signals after substraction of positive signals from the control staining (not shown), and thus the same ratio of added *Nitrosomonas* spp. was recovered.

Distribution of serotypes A10 and A13 in different sewage plants. Activated-sludge samples from six domestic sewage

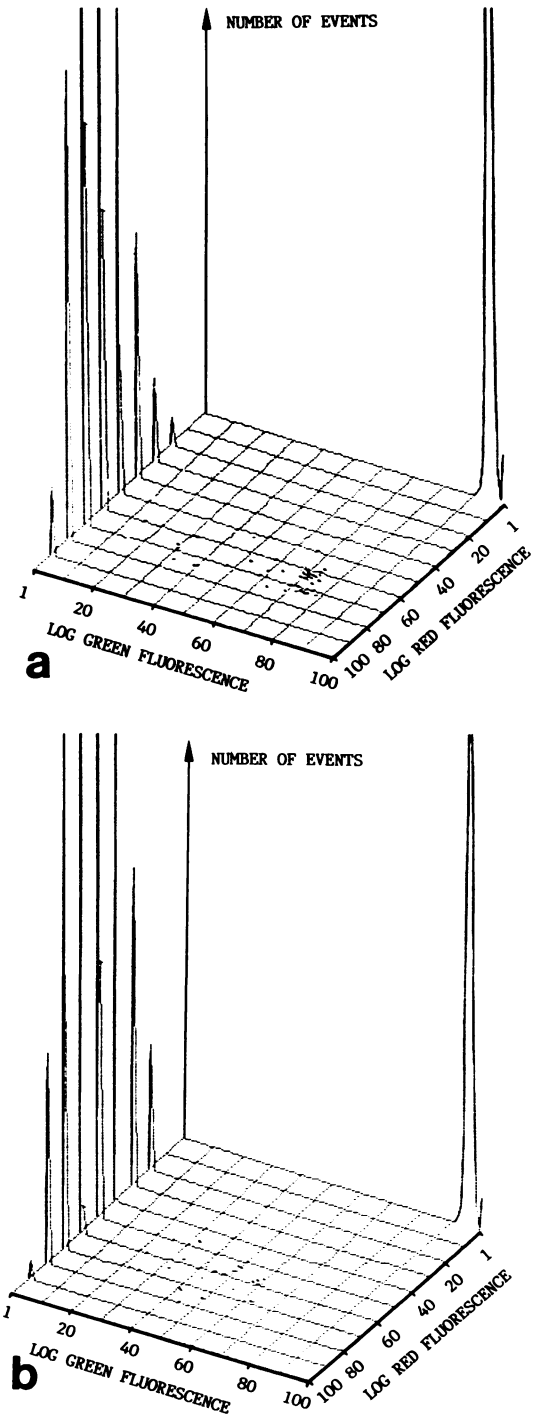

FIG. 2. FCM study of activated sludge stained with antiserum A13 (a) or control serum (b). The cluster above channel 60 of green fluorescence (center of the histogram) accounts for 0.5% of all bacteria. (b) After substraction of the concentration of "immunofluorescent" bacteria detected in the same area with control serum (0.1%), a concentration of 0.4% *Nitrosomonas* serotype A13 was found with this technique. A total of 50,000 bacteria were analyzed.

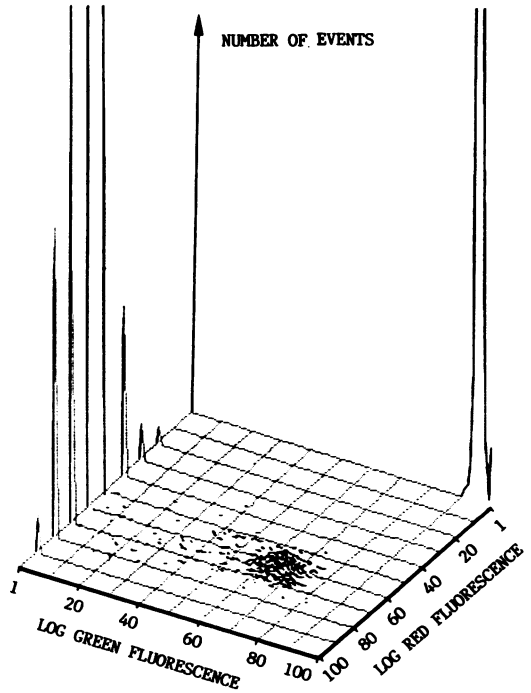

FIG. 3. FCM study of activated sludge enriched with a 2.5% concentration of *Nitrosomonas* serotype A13 of total bacteria in the sludge and labeled with the antiserum against this serotype. The cluster above channel 60 of green fluorescence accounts for 2.9% of all bacteria after substraction of control staining (data not shown). The large peak in channel 95 of green fluorescence (far right) represents microspheres, used as calibration particles.

plants in various regions of Germany and Switzerland and from two industrial sewage plants were analyzed by FCM with the antiserum against *Nitrosomonas* serotype A13, and the bacterium was always detected where nitrification occurred (Table 1).

The same sewage plants were also analyzed with the antiserum against *Nitrosomonas* serotype A10, and only in three of them were significant amounts of these bacteria found. Over a period of 6 months, all measurements with

TABLE 1. Activities of ammonia-oxidizing bacteria and concentrations of *Nitrosomonas* serotypes A10 and A13 determined by FCM in various sewage plants

Sewage plant	Date	Concn of *Nitrosomonas* (% of total bacteria)		Activity (nmol/s per g, wet wt)	Nitrification rate (% of ammonia removed)
		Serotype A10	Serotype A13		
Bad Wimpfen, F.R.G.	9-22-89	2.0	0.5	6.9	26
Biberach, F.R.G.	11-23-89	1.6	1.4	4.5	77
Horb, F.R.G.	9-23-89	<0.1	0.4	4.2	53
Calw, F.R.G.	9-22-89	0.8	0.4	5.1	92
Berlin	5-3-89	<0.1	1.7	7.8	>95
Zurich, Switzerland	5-3-89	<0.1	1.5	ND[a]	>95
Industrial sewage plant I	9-19-89	<0.1	<0.1	0	0
Industrial sewage plant II	9-19-89	<0.1	0.2	1.4	66

[a] ND, Not determined.

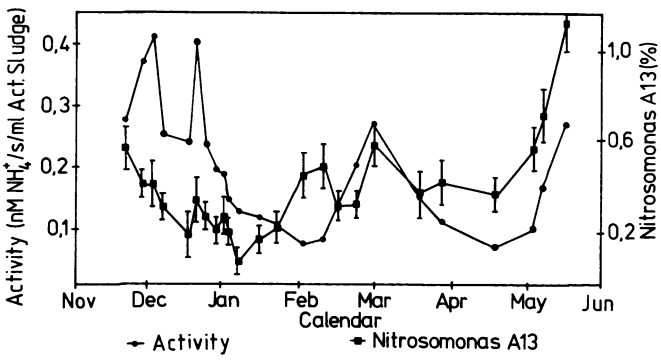

FIG. 4. Variation of percentage of *Nitrosomonas* serotype A13 determined by FCM analysis over a period of 6 months. This is shown in relation to the activity of ammonia-oxidizing bacteria in activated sludge, measured by the described in vitro assay (see text). Activity is expressed as the turnover (nanomoles) of ammonia per second per milliliter of activated sludge.

activated sludge from the sewage plant Heidelberg revealed that bacteria of this serotype were not detectable by FCM without preenrichment, but were present in MPN tubes at lower sample dilutions. In contrast, *Nitrosomonas* serotype A13 was always detectable in activated sludge by FCM and always occurred in the positive MPN tubes of the highest dilution for a period of 2 years.

Concentrations of ammonia-oxidizing bacteria in the sewage plant Heidelberg were determined monthly by the MPN technique. Numbers in the range of 1×10^4 to 100×10^4 cells per ml were found. This analysis was also performed with sludge from three other sewage plants, with similar results.

Comparison of activity and concentration of ammonia-oxidizing bacteria. Parallel to the analysis by FCM, activities of ammonia-oxidizing bacteria were measured as described in Materials and Methods and calculated from the linear rate of nitrite accumulation during the initial 10 min of the test. Control experiments with this assay system revealed that (i) the increase of nitrite in the presence of 20 mM sodium chlorate was linear for at least 30 min; (ii) the increase of nitrite was proportional to the amount of activated sludge; (iii) in the presence of 20 mM sodium chlorate and 5 mg of *N*-allylthiourea, a selective inhibitor of ammonia oxidizers (29, 32), per liter there was no accumulation of nitrite; and (iv) in the presence of 20 mM sodium chlorate, 5 mg of *N*-allylthiourea per liter, and 1 mM nitrite, the concentration of nitrite remained constant for more than 30 min.

Over a period of 6 months, we compared the ammonia-oxidizing activity of activated sludge with the concentration of *Nitrosomonas* serotype A13, determined by FCM analyses (Fig. 4). With one exception, during February 1989, activities of ammonia-oxidizing bacteria and concentrations of *Nitrosomonas* serotype A13 decreased and increased in parallel.

If *Nitrosomonas* serotype A13 is the major ammonia-oxidizing population in the sludge, the specific activity (k_0) of a single bacterium can be calculated by the formula: k_0 = (activity per milliliter)/number of *Nitrosomonas* A13 per milliliter. With this calculation, we determined the specific activity to be in the range of 0.05 to 0.4 pmol/cell per h.

For comparison, we determined the specific activity of *Nitrosomonas* serotype A13 in a pure culture. In both cases, when grown in either MPN medium or sterile filtered supernatant of activated sludge, which may contain growth fac-

tors, specific activities of 0.02 pmol/cell per h were calculated from the increase of nitrite.

DISCUSSION

Two serotypes of *Nitrosomonas* spp., denoted A10 and A13, were isolated from samples of the sewage plant Heidelberg and were detected and quantified in activated sludge by FCM. In contrast to the detection of ammonia-oxidizing bacteria by the MPN technique, which requires incubation times of at least 1 month, this technique enabled us to detect and enumerate these bacteria within several hours. Fluorescent-antibody techniques exist for the identification of *Nitrosomonas* spp. under the fluorescence microscope (30, 31). Major limitations to the application of this method to activated sludge are problems with background fluorescence, which are possibly due to strong absorption of the antibodies to the carbohydrate matrix of the sludge. Furthermore, there is a certain amount of bacteria in this environment against which even very young rabbits have already formed antibodies. Visual resolution of these bacteria and background fluorescence from immunofluorescent *Nitrosomonas* spp. is difficult, and thus fluorescence microscopy is not a reliable technique for the quantitative estimation of *Nitrosomonas* spp. in activated sludge.

FCM allows the fluorescence detection of individual cells in suspension as they travel through the laser beam. The DNA content of each cell can be determined by the red fluorescence of the DNA-propidium iodide complex. Thus, particles with a DNA content in the range of bacteria can be selected for further analysis, and fragments from sludge without DNA can be excluded. Studies with pure cultures revealed that the intensity of green fluorescence of immunofluorescently labeled bacteria of *Nitrosomonas* serotype A13 was above a certain channel (channel 60) of green fluorescence. This channel was set as a threshold for the detection of *Nitrosomonas* spp. in activated sludge.

Concentrations of bacteria against which rabbits had formed antibodies prior to immunization were determined by measuring samples labeled with control serum and substracted from concentrations of bacteria labeled with antiserum. The use of monoclonal antibodies will circumvent the latter problem in the future.

A small proportion of *Nitrosomonas* serotype A13 grown in pure culture remained unstained with the homologous antiserum (10%). This may be due to dead bacteria, which have lost their surface antigens. *Nitrosomonas* serotype A13 could be detected in all examined sewage plants where nitrification occurred. Serotype A10 only occurred in two domestic sewage plants and was under the detection limit (<0.1% of the total bacteria) of FCM in the others. In parallel, the number of ammonia-oxidizing bacteria was determined in the activated sludge of the sewage plant Heidelberg each month over a period of 2 years by the MPN technique. In the positive MPN tubes of the highest dilution, *Nitrosomonas* serotype A13 was always detected by FCM analysis. Efforts to isolate more species and serotypes of ammonia-oxidizing bacteria from MPN tubes at high dilutions failed. These results indicate that *Nitrosomonas* serotype A13 is an important ammonia-oxidizing bacterium in sewage plants with nitrification. Furthermore, concentrations of *Nitrosomonas* serotypes A13 and A10 were in the range of 0.2 to 2% in all nitrifying sewage plants. These values are in agreement with estimations and measurements of other authors, which reveal that the maximal concentration of ammonia oxidizers does not exceed 1 to 2% of the total bacteria in activated sludge (for a review, see reference 23).

Nevertheless, other species of ammonia-oxidizing bacteria or *Nitrosomonas* serotypes might occur in activated sludge from sewage plants and might even be more important for the nitrification process, but more difficult to isolate. It has to be considered that the media used for the MPN technique might select for certain ammonia oxidizers (5). Thus, other authors found a greater variety among ammonia-oxidizing bacteria (6, 30).

We further compared the activity of ammonia-oxidizing bacteria in sludge with the concentration of *Nitrosomonas* serotype A13. These results revealed that the bacterium might be used as an indicator for changes in the potential of the sewage plant activated sludge to oxidize ammonia (Fig. 4).

Under the assumption that *Nitrosomonas* serotype A13 is the dominant ammonia-oxidizing bacterium in the sewage plant at Heidelberg, its specific activity was calculated from the results of FCM and activity measurements. This activity varied between 0.2 and 0.4 pmol/cell per h from December to January and decreased to 0.05 to 0.2 pmol/cell per h during February to March. This can be explained either by changes in the vitality of the microorganism or by the presence of other ammonia oxidizers, which were more dominant during December and January but did not grow in the chosen MPN medium.

The specific activity of *Nitrosomonas* serotype A13 in pure culture during exponential growth was 0.02 pmol/cell per hour, which is in the same range as values described in the literature (2, 4), but 2 to 20 times lower than specific activities calculated for the sludge (see above).

Thus, serotype A13 might represent only 10% of all ammonia oxidizers in the activated sludge, or the activities of ammonia oxidizers in the sewage plant are 10 to 20 times higher than in pure culture, or our estimations of the total bacterial content of sludge are too low by a factor of 10. We propose that the activity of *Nitrosomonas* serotype A13 was lower in pure culture than in the sewage plant, because ammonia oxidizers probably need their natural environment for optimal activity, i.e., growth in flocs and interaction with other bacteria (17, 18).

The concentrations of ammonia oxidizers determined by the MPN technique were 1×10^4 to 100×10^4 cells per ml and are probably 20 to 2,000 times too low. Other authors found similar counting efficiencies (4, 10).

We conclude that FCM is a useful technique to detect and measure concentrations (higher than 0.1% of total bacteria) of ammonia-oxidizing bacteria in samples from activated sludge and possibly from other environments. These results can be combined with activity measurements, which might enable us to estimate the importance of these organisms. Furthermore, rapid techniques for the determination of concentrations and activities of nitrifying bacteria in activated sludge might help to elucidate the growth parameters for these bacteria in this environment under various technical conditions. Some technical parameters that influence the growth of these organisms are oxygen concentration and temperature in the aeration basin, sludge management, and composition of the incoming wastewater. Knowledge of the exact relation between biological and technical parameters might provide engineers with new data to maintain a better and more stable nitrification.

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