APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1982, p. 210–217
0099-2240/82/010210-08$02.00/0

Vol. 43, No. 1

Feasibility of ^{55}Fe Autoradiography as Performed on N_2-Fixing *Anabaena* spp. Populations and Associated Bacteria

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Received 11 June 1981/Accepted 28 August 1981

^{55}Fe emits low-energy X rays and Auger electrons by electron capture decay. Auger electrons are useful for autoradiographic examination of ^{55}Fe incorporation among microbial communities. Attainable resolution, in terms of silver grain deposition, is excellent and comparable to ^{3}H. Two known Fe-demanding processes, photosynthetic CO_2 fixation and N_2 fixation, were examined by autoradiography of *Anabaena* populations. During photosynthetically active (illuminated) N_2-fixing periods, biological incorporation of $^{55}FeCl_3$ by vegetative cells and heterocysts was evident. When N_2 fixation was suppressed by NH_4^+ additions, heterocysts revealed no incorporation of ^{55}Fe. Conversely, when N_2-fixing *Anabaena* filaments were placed in darkness, ^{55}Fe incorporation decreased in vegetative cells, whereas heterocysts showed sustained rates of ^{55}Fe incorporation. Bacteria actively incorporated ^{55}Fe under both light and dark conditions. The chelated (by Na_2-ethylenediaminetetraacetate) form of $^{55}FeCl_3$ was more readily incorporated than the nonchelated form. Furthermore, abiotic adsorption of ^{55}Fe to filters and nonliving particles proved lower when chelated ^{55}Fe was used in experiments. ^{55}Fe autoradiography is useful for observing the fate and cellular distribution of various forms of Fe among aquatic microbial communities.

^{55}Fe is a potentially useful radioactive isotope of iron, since it has a reasonably long half-life (approximately 2 years) and measurable emission products, in the form of low-energy X rays and electrons (5). ^{55}Fe decays by electron capture, where the nucleus captures an electron from the inner electron shell, which is then filled by an outer electron (1). The sequence of events releases energy in the form of X rays or by the ejection of an electron from another shell. This form of electron ejection is termed Auger emission. Auger electrons are potentially useful in the autoradiographic detection of ^{55}Fe, having energies of 5 to 6 keV and 0.5 to 0.6 keV, similar to ^{3}H β^- decay energies (13).

Numerous studies have proven the value of ^{3}H as a tracer applicable to autoradiography, because the low-energy β^- emissions characteristic of this isotope yield extremely high-resolution autoradiographs. Orlic (10) applied ^{55}Fe in high-resolution autoradiographic studies of erythrocytes, and Parry and Blackett (13) demonstrated that ^{55}Fe could yield resolution similar to ^{3}H in electron microscopic autoradiographic studies of erythroid cells. After these studies, ^{55}Fe has received increased application in medical and physiological studies. The utilization, localization, and metabolism of Fe is of obvious importance among microbial communities as well, for it is known that numerous photosynthetic and heterotrophic microorganisms have substantial Fe requirements. In aquatic ecosystems, Fe availability has been shown to limit primary productivity (6, 7), further evidence of the importance of Fe in controlling microbial metabolism in nature.

N_2-fixing cyanobacteria are of particular interest in considering iron requirements, since both their photosynthetic and N_2-fixing pathways require Fe to properly function (4) and recent work by Peterson and Wolk (14) used ^{55}Fe as a label for localizing nitrogenase in *Anabaena variabilis*. Accordingly, the filamentous N_2-fixing cyanobacterium *Anabaena* sp. was chosen as an organism for evaluating the usefulness of ^{55}Fe in microautoradiographic studies. This study describes the fate and distribution of ^{55}Fe among natural and isolated *Anabaena* populations.

MATERIALS AND METHODS

An ^{55}Fe-$FeCl_3$ solution in 0.1 M HCl, having a specific activity of 78.5 mCi · mg of Fe^{-1}, was obtained from Amersham Corp., Arlington Heights, Ill. Chelated and nonchelated solutions of $^{55}FeCl_3$ were used. Disodium ethylenediaminetetraacetate (Fisher Scientific Co., Fair Lawn, N.J.) was added to $^{55}FeCl_3$ in a molar ratio of 2:1 to produce chelated ^{55}Fe. Nonchelated ^{55}Fe solutions were made up by diluting the original solution with distilled deionized water. All solutions were heat sterilized and stored in acid-cleaned and deionized water-washed 125-ml polycarbonate flasks with Nalgene screw caps.

Both natural populations and an isolated species of *Anabaena* were used for autoradiographic experiments. *Anabaena spiroides* was the dominant bloom-forming species in Thompson Lake near Toronto, Canada. This small (8 hectares), highly eutrophic lake was periodically sampled during a 4-month summer bloom in 1978. *A. spiroides* dominated the phytoplankton community of the lake, accounting for 82 to 97% of the total phytoplankton biomass during this period (8). In situ acetylene reduction assays revealed high rates of N_2 fixation, attributable to *A. spiroides* (8). Axenic and nonaxenic cultures of *Anabaena oscillarioides* were isolated from the Waikato River, New Zealand, and kindly donated by Y. K. Lam, Ministry of Works, Hamilton, New Zealand (Y. K. Lam, Ph.D. thesis, University of Auchland, New Zealand, 1978). Batch cultures were grown and maintained in N-free Chu-10 medium (2) on a shaker table illuminated by 2,000-lx cool-white fluorescent lights with a 14-h light/10-h dark cycle. The culture temperature was 24°C.

Adsorption of $^{55}FeCl_3$ and other trace elements to glassware proved to be problematic. As a result, polycarbonate incubation flasks were preferred. All ^{55}Fe incubations were conducted in 250-ml screw-cap transparent acid-cleaned and deionized water-washed polycarbonate flasks (Corning Glass Works, Corning, N.Y.). Flasks were clamped onto a gyratory shaker during incubations.

Incubations were started by transferring 150 ml of *Anabaena* suspension and by dispensing 10 μCi of $^{55}FeCl_3$ into a flask and loosely sealing the cap on each flask. Abiotic isotope adsorption to particulate material was detected by adding buffered Formalin (pH 8.0) (Fisher Scientific Co.) at a final concentration of 3% before isotope addition. Progressive incorporation of ^{55}Fe into particulate matter was monitored by the use of liquid scintillation spectrometry, which was capable of detecting Auger electron emissions at approximately 45% efficiency, as determined by the use of internal ^{55}Fe standardization. All incubations were done in triplicate. Both chelated and unchelated forms of ^{55}Fe had a tendency to adsorb to filters used to concentrate *Anabaena* spp. and other forms of particulate material. Millipore HA type filters (Millipore Corp., Bedford, Mass.), which previously proved useful for ^{3}H and ^{14}C autoradiography (11, 12), adsorbed unacceptable amounts of ^{55}Fe, no matter what type of pretreatment was applied to eliminate this problem. Nuclepore (Nuclepore Corp., Pleasanton, Calif.) polycarbonate membrane filters proved more useful. Two pretreatment techniques were routinely used to minimize ^{55}Fe adsorption to these filters. Filters could be soaked in either a 0.001 M $FeCl_3$ solution or a 0.001 M disodium ethylenediaminetetraacetate solution for 24 h at a pH of 5.00 before filtration. With this pretreatment, filter adsorption of $^{55}FeCl_3$ added to particle-free lake or culture water proved to be less than 5% of the incorporation by particulate matter. This level of filter adsorption proved satisfactory for distinguishing background radiation from incorporation by particulate matter in autoradiographs.

Autoradiographs were prepared by gently filtering (200 torr) variable amounts of lake or culture water through 25-mm Nuclepore filters with a porosity of 0.2 μm. Volumes of ^{55}Fe-incubated material were variable due to a range of particle concentrations in the variety of samples examined. Since densities of particulate material on filters can affect both background exposures and the ability of the investigator to discern individual particles, optimal particle concentrations were initially determined by trial autoradiographs. High particle densities often lead to unacceptably high background exposure; hence, a low particle density was preferred.

After particle filtration, filters were rinsed with a 10-ml volume of 0.001 M Na_2-ethylenediaminetetraacetate (pH 8.00) to minimize ^{55}Fe adsorption to particulate matter, followed by a 30-s (10-ml) rinse of 2% buffered Formalin (pH 8.0). Excess Formalin was removed by a 10-ml rinse of 0.2-μm-filtered culture or lake water. Filters were then air dried in a desiccator and mounted, face up, with an optically transparent adhesive on clean microscope slides. Several adhesives proved useful for mounting, including all clear acetone-based adhesives, Elmer's wood glue (Borden, Inc., Columbus, Ohio) and thin applications of rubber cement. Ideally, applications of adhesives, covering only the edges of filters (leaving the middle of the filters free of adhesives), yield the best optical result. In total darkness, filters mounted on slides were dipped in Kodak NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, N.Y.), which was diluted 1:1 with distilled water and heated to 40°C. The dilution with water resulted in a uniformly thin coating (approximately 3 to 5 μm in thickness), as determined in earlier work with ^{14}C- and ^{3}H-labeled samples (11, 12). Dipped slides were packed in desiccated light-tight slide boxes and stored at 4°C until development. Several sets of replicate filters were dipped and exposed for each experiment to arrive at the proper exposure. Most samples revealed useful exposures if left to expose for 2 to 4 weeks in slide boxes. Background exposures and the possibilities of chemography were tested by preparing ^{55}Fe-free autoradiographs of all samples.

Autoradiographs were developed for 2 min in full-strength Kodak D-19 developer, using a slide-staining rack for holding autoradiographic slides. Slides were transferred to a distilled water stop bath for 30 s and placed in Kodak fixer for 2 min. Slides were then carefully washed in three baths of distilled water, transferring the staining rack to each bath, every 5 min. Minimal agitation during all processing steps minimized separation of filters from the emulsion. After the slides were washed, they were hung to air dry.

All autoradiographs were viewed at ×1,000, using a Zeiss phase-contrast microscope (Carl Zeiss Inc., Silver Spring, Md.), with an oil-immersion objective. Slides viewed in this manner could be stored sideways in a dustproof slide box, allowing excess oil to run off the slides onto a tissue placed on the bottom of the box. Oil should not be wiped off the emulsion under any circumstance, since this will damage and remove the emulsion.

RESULTS AND DISCUSSION

General characteristics of ^{55}Fe autoradiographs. Per amount of radioactivity added, ^{55}Fe led to approximately one-half to one-third of the silver halide exposures as ^{3}H (Table 1). The cause of lower exposure characteristics was not established here; however, since ^{3}H emits β^-

TABLE 1. Exposure intensity, in terms of silver grain density per filament, for ^{3}H- and ^{55}Fe-labeled *A. oscillarioides* filaments[a]

Isotope added	Expt no.	Absolute activity (dpm) on filter[b]	No. of silver grains per filament (minus background)
^{3}H	1	20,155	35.7
^{3}H	2	19,858	34.2
^{3}H	3	20,320	34.8
^{55}Fe	1	31,056	14.1
^{55}Fe	2	28,699	13.8
^{55}Fe	3	29,722	14.5

[a] An average of 100 filaments per filter were examined by random selection. [U-^{3}H]sodium acetate and chelated $^{55}FeCl_3$ were the respective isotope forms. Filaments were taken from a single axenic culture. As a result, identical filament densities per unit volume of filtered water were present in all samples. After a 1-h incubation with each isotope, 2 ml of culture volume was filtered onto Nuclepore filters.

[b] Absolute activity was determined by liquid scintillation counting and corrected for filter adsorption. All autoradiographs were exposed for 2 weeks.

particles and ^{55}Fe emits lower energy Auger electrons and some X rays, it might be expected that a different exposure intensity would result from the use of these contrasting isotopes. For practical application, it means that ^{55}Fe autoradiographs must be exposed two to three times as long, per number of disintegrations per minute, than ^{3}H autoradiographs. Increased exposure periods can be compensated for to some extent by increasing the amount of ^{55}Fe added. However, increased ^{55}Fe concentrations also lead to higher amounts of ^{55}Fe adsorption to filters (in terms of absolute counts), hence increasing background exposures. Therefore, optimal exposure times must be sought with these factors in mind. Extreme care must also be taken to minimize natural background exposures commonly attributable to cosmic radiation, static electricity, light leaks, and X rays, since long exposure times are required. Storage of exposing slides in a refrigerator at approximately 4°C helps minimize background exposures. Packing slide boxes in lead containers should help as well, although this was not attempted here.

In general, ^{55}Fe exposures yielded resolution comparable to ^{3}H, making it a useful isotope for microautoradiographic studies. Parry and Blackett (13) came to similar conclusions when using erythroid bone marrow cells. The mean distance at which ^{55}Fe exposures could be detected from ^{55}Fe-labeled bacteria proved to be 1.5 μm, which was similar to results obtained with ^{3}H-labeled cells (Fig. 1). Again, these results confirm several earlier determinations of ^{55}Fe resolving power (10, 13).

Adsorption of ^{55}Fe to filters and abiotic adsorption of ^{55}Fe to particulate matter proved problematic in isolated cases, but not in most cases (Table 2). When biological incorporation was evident, and Fe demands were high, abiotic factors were negligible. This was the case in about 90% of all samples prepared. However, when Fe demands were very low, as was the case with nonphotosynthetic (dark) *Anabaena* filaments, it became difficult to separate abiotic adsorption from any residual biological incorporation. In such cases, it could only be concluded that ^{55}Fe incorporation was nondetectable.

Chelated versus nonchelated ^{55}Fe additions. When added in trace concentrations, some differences were observed between chelated and nonchelated $^{55}FeCl_3$ adsorption and incorporation. Chelated $^{55}FeCl_3$ proved to be physically adsorbed to nonliving surfaces and filters. In Thompson Lake water, filter adsorption of ^{55}Fe proved to be 2.5% of the total incorporation when chelated $^{55}FeCl_3$ was added; when nonchelated $^{55}FeCl_3$ was added to the same water samples, as much as 35% of the total incorporation proved to be filter adsorption. Similar results were obtained when abiotic adsorption to particulate matter was determined. Formalin-killed samples revealed that 7.5% of the $^{55}FeCl_3$ incorporation was abiotic when chelated $^{55}FeCl_3$ was added; 29% of the incorporation was abiotic when nonchelated $^{55}FeCl_3$ was added.

Both *Anabaena* spp. and associated bacteria were able to actively incorporate chelated and

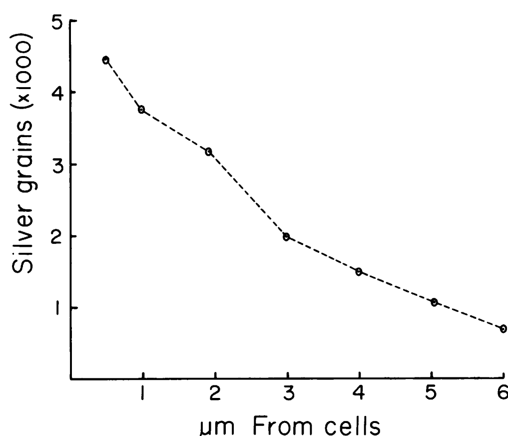

FIG. 1. Frequency of distances away from bacteria at which silver grain deposition can be detected in an ^{55}Fe autoradiograph. Silver grains counted represent the total tally of grains exposed around bacteria detected in 20 randomly chosen microscope fields. Bacteria and silver grains were viewed at ×1,000, using oil-immersion phase-contrast optics.

TABLE 2. Filter adsorption, abiotic adsorption to particulate matter, and biological incorporation of chelated and nonchelated $^{55}FeCl_3$[a]

Sample	Absolute activity (dpm) for[b]:		
	Filter adsorption	Abiotic adsorption	Biological incorporation
Thompson Lake			
Chelated	695 ± 251	2,049 ± 341	30,150 ± 755
Nonchelated	12,950 ± 597	10,127 ± 338	37,065 ± 944
A. oscillarioides			
Chelated	970 ± 253	1,744 ± 241	17,597 ± 609
Nonchelated	2,435 ± 378	3,998 ± 614	21,793 ± 1,025

[a] Results are from 10 ml of lake water or culture filtrations.

[b] Results are the averages of triplicate samples (± standard error) as determined by liquid scintillation counting. Counting efficiencies were determined by use of a calibrated internal ^{55}Fe standard.

nonchelated $^{55}FeCl_3$. However, in both lake water and culture media, chelated $^{55}FeCl_3$ was more readily incorporated. The addition of $^{55}FeCl_3$ in both forms proved to be useful in discriminating N_2-fixing from non-N_2-fixing *A. oscillarioides*. During illuminated N_2-fixing periods, *A. oscillarioides* revealed ^{55}Fe labeling in both vegetative (photosynthetic) cells and heterocysts (Fig. 2). In the presence of NH_4^+, which was able to suppress N_2 fixation after 6 h, subsequent ^{55}Fe additions were rapidly incorporated by vegetative cells but were less actively incorporated by heterocysts (Fig. 3). Aerobic N_2 fixation has been attributed to the heterocysts by several workers (3, 17), and N_2 fixation, specifically nitrogenase, has a strong requirement for Fe (4, 14); the autoradiographic evidence provided here substantiates these findings. Since various enzymes and cofactors involved in photosynthesis also have strong Fe requirements, the cessation of photosynthesis would lead to reduced ^{55}Fe incorporation in vegetative cells. Accordingly, an N_2-fixing *A. oscillarioides* culture was transferred from illu-

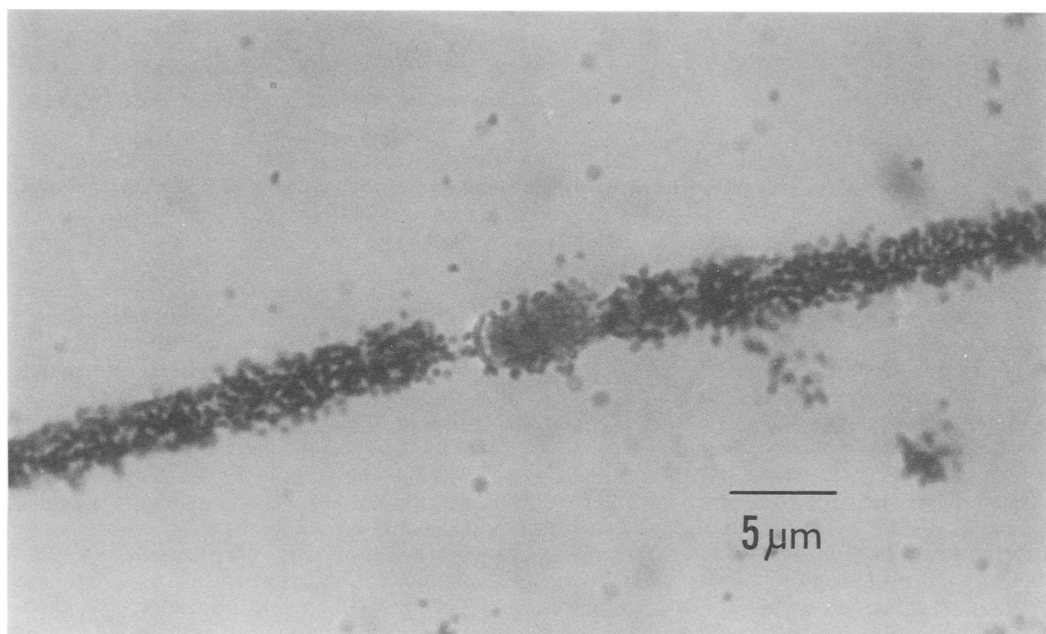

FIG. 2. ^{55}Fe (added as chelated $^{55}FeCl_3$) incorporated in a filament of axenically grown *A. oscillarioides*. Filaments were illuminated and fixing N_2. Silver grain deposition can be seen as dark grain patterns superimposing *Anabaena* filaments. Heavy deposition patterns can be seen covering both vegetative cells and heterocysts. The frequencies of labeled vegetative cells and heterocysts were 93 and 85%, respectively. The sample was prepared 2 h after ^{55}Fe addition.

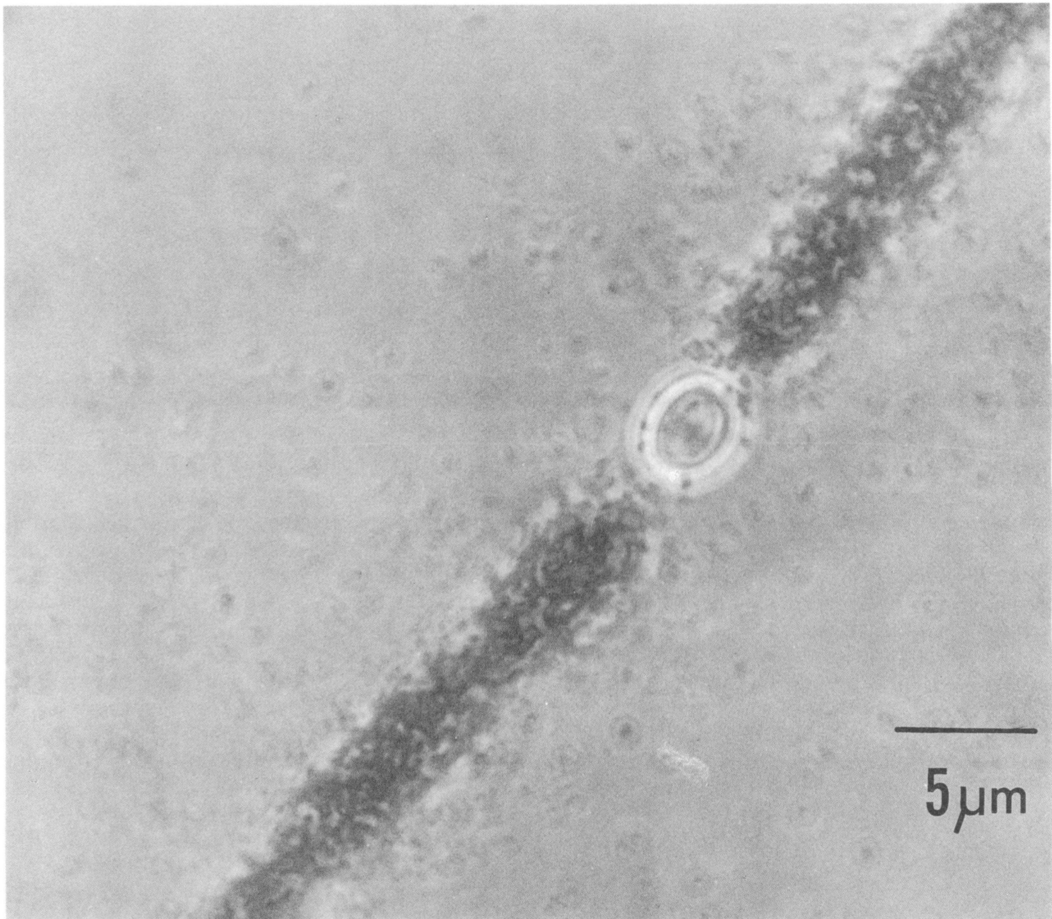

FIG. 3. Chelated $^{55}FeCl_3$ incorporation in an illuminated filament of *A. oscillarioides* grown on NH_4^+ for 6 h before ^{55}Fe addition. The addition of NH_4^+ effectively suppressed N_2 fixation. This autoradiograph reveals heavily labeled vegetative cells, but virtually unlabeled heterocysts. The frequencies of labeled vegetative cells and heterocysts were 91 and 11%, respectively. The sample was prepared 2 h after ^{55}Fe addition.

minated to dark conditions and incubated with chelated $^{55}FeCl_3$. The results represented a dramatic contrast in labeling compared with illuminated conditions. Vegetative cells revealed only traces of ^{55}Fe incorporation, whereas the heterocysts were the most heavily labeled cells in the filaments (Fig. 4).

Bacteria, either free-floating or attached to *Anabaena* filaments, consistently and actively incorporated ^{55}Fe (Fig. 5). No detectable differences between illuminated and dark conditions were observed in terms of ^{55}Fe incorporation per cell, indicating that heterotrophic processes might be dictating Fe utilization. As with *Anabaena*, bacteria were able to utilize chelated $^{55}FeCl_3$ more readily than nonchelated $FeCl_3$. Natural bacteria found in Thompson Lake water were often located in clumps or small colonies (Fig. 5). Bacteria residing in such clumps actively incorporated $^{55}FeCl_3$ in the chelated form, but failed to incorporate $^{55}FeCl_3$ in the nonchelated form.

In both chelated and nonchelated forms, $^{55}FeCl_3$ was often detected in nondescript clumps ranging from 2 to 10 μm across. These clumps did not appear to contain either bacteria or algae. Subsequent observations made with epifluorescent microscopy also failed to lead to distinguishable microorganisms. Approximately 20% of the ^{55}Fe labeling associated with clumps appeared to be abiotic, as determined by comparing Formalin-treated cells with untreated samples. Closer examinations of clumps revealed that they were often associated with *Anabaena* filaments. Subsequent microscopic counts of clumps revealed that approximately

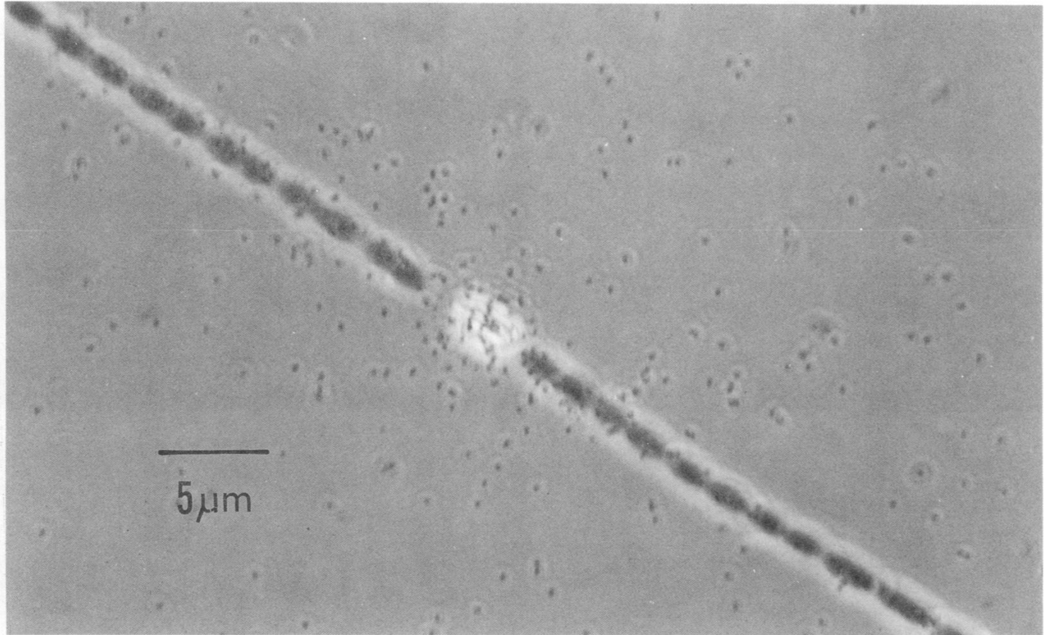

FIG. 4. Chelated $^{55}FeCl_3$ incorporation in N_2-fixing *A. oscillarioides* grown under complete darkness. Under these conditions, vegetative cells were not active in ^{55}Fe incorporation. Heterocysts showed low but sustained ^{55}Fe incorporation. The sample was prepared 2 h after ^{55}Fe addition.

55% of all clumps were attached to *Anabaena* filaments, the remainder being detached. Samples treated with Formalin failed to reveal labeled clumps on *Anabaena* filaments. A test for background exposures and chemography indicated that clumps were not due to chemical exposure of the emulsion.

Since it is known that *Anabaena* spp. and other cyanobacterial and bacterial species actively excrete mucoid slime material composed of polysaccharides and to a lesser extent peptides (15, 16), it is likely that the clumps observed may constitute such materials labeled with ^{55}Fe. The fact that ^{55}Fe labeling of clumps appears to be largely biologically mediated suggests that $^{55}FeCl_3$ is actively incorporated from lake and culture water and biochemically combined or excreted along with mucoid materials. Two modes of biologically active ^{55}Fe-mucoid combinations are possible: (i) ^{55}Fe is actively incorporated and subsequently excreted after combining with precursors of extracellular slimes, and (ii) ^{55}Fe is not incorporated or transported across the cell walls, but rather chemically combined or bound to extracellular slimes as they appear outside the cell wall. Whichever is the case, the formation of ^{55}Fe clumps can be arrested by Formalin additions. Clearly, further high-resolution ^{55}Fe autoradiography in combination with chemical separation of cell wall and slime constituents would help clarify the alternative possibilities. Elucidation of the process of ^{55}Fe slime and clump production may be of central importance in clarifying how cyanobacteria and bacteria bind Fe compounds through extracellular production of slimes. The above observations may be relevant to the observed production of powerful hydroxymate Fe-binding chelators known to be produced by cyanobacteria (9).

This study demonstrated the usefulness of ^{55}Fe autoradiography as applied with the dipping-emulsion technique. The high resolution attained with ^{55}Fe makes this a feasible isotope for visualizing cellular localization and transformation of Fe-containing nutrients and organic compounds. Initial applications of ^{55}Fe autoradiography have shown localized regions of high Fe demand in cyanobacteria, all in agreement with earlier biochemical and physiological determinations (4, 14). This method is capable of visually discriminating the fate of Fe compounds in natural waters among diverse microbial communities. ^{55}Fe autoradiography may also be useful in detecting and localizing sites of Fe binding not directly mediated by abiotic processes.

The method of preparing and observing ^{55}Fe autoradiographs is not without problems. Abiotic adsorption to filters and particulate matter constitutes a significant fraction of the biological

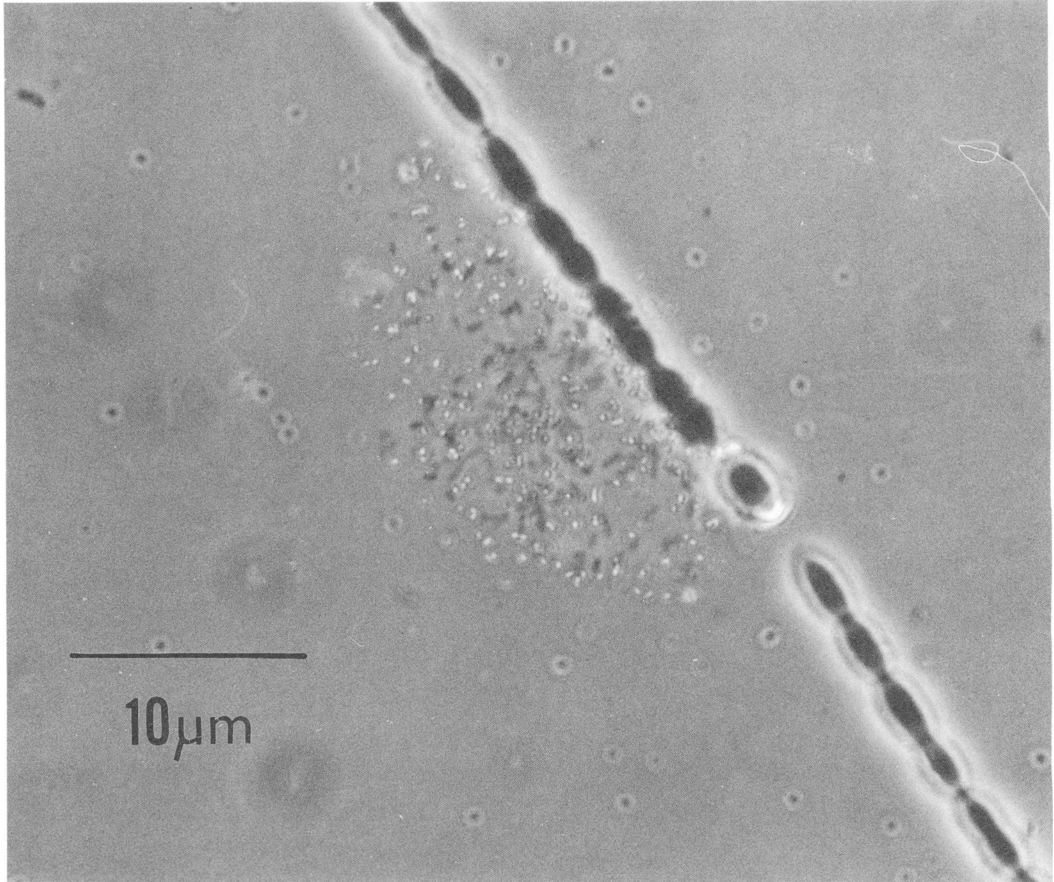

FIG. 5. Bacterial incorporation of chelated $^{55}FeCl_3$. The sample was incubated in total darkness, and autoradiographs were prepared and incubated 2 h after ^{55}Fe addition. The sample was taken from a 1-m depth and incubated in situ in Thompson Lake, Ontario, Canada. At the time of the sampling, the microbial community was dominated by clumped bacteria. Clumps were often associated with *Anabaena* filaments. In this photograph, bacteria appear darkened and exposed silver grains appear as light dots; this is due to phase-contrast image enhancement, needed to reveal both bacteria and silver grains. The diameter of this bacterial clump is approximately 10 µm.

incorporation. As a result, care must be taken to separate and identify diverse Fe-binding processes. Perhaps with the use of more diverse chelating agents and purified natural Fe chelators, problems in discriminating biotic from abiotic interactions of Fe with particles and macromolecular compounds will be facilitated.

ACKNOWLEDGMENTS

Field and laboratory assistance by T. P. Murphy, P. E. Kellar, D. B. Shindler, and D. R. S. Lean was appreciated, and comments on the preparation of the manuscript by W. Sunda and E. White proved invaluable. J. Garner aided with the preparation of the manuscript.

This research was funded by the Inland Waters Directorate/Environment Canada, the Department of Scientific and Industrial Research, Ecology Division, New Zealand, and the office of Water Research and Technology, U.S. Department of the Interior/Water Resources Research Institute of the University of North Carolina (grant B-127-NC[b]).

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