THE ISOLATION AND CHARACTERIZATION OF NANNOBACTERIA

THESIS

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ABSTRACT

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With improvements in technology in recent years, new realms of images are being discovered by high power microscopy. One such discovery has been of very small structures resembling bacteria, only much smaller, at approximately $1/1000^{th}$ the volume. These structures have been noted in many images of waters and minerals from places such as hot springs, rivers and even a Martian meteorite. They have been named "Nannobacteria" and hypothesized to play a role in the formation of minerals such as calcite and aragonite. This research attempts to investigate whether or not these structures are in fact biological. Attempts to culture nannobacteria on TSA, R2A, SDA, DMEM and RPMI in environments of 25°C, 37°C, 37°C with CO_2, and 45°C, have been made. Experiments to rule out the possibility that they are biological artifacts, such as bacterial fragments have also been performed.

INTRODUCTION

Scientists have traditionally considered the size of a bacterium to fall within the range of 0.02 to 400 μm^3 (23). The upper limits were suggested by the level of diffusion that can occur across a membrane and into a large cell. However, recent findings have pushed those upper limits to include a very large bacterium, *Epulopiscium fishelsoni* (3), some of which have been found to be larger than 600 μm by 80 μm. The lower limits (0.02 μm^3) were based on how big a cell must be in order to house the appropriate machinery necessary to carry out the functions for living. A genome (genetic material), ribosome (site for protein synthesis) and protein must be present (23) for an organism to be capable of independent life as we know it. Studies by Torrella and Morita have isolated "dwarf" bacteria, or ultramicrobacteria, from seawater that are below the size of 0.3 μm in diameter (36). This small size is attributed to a starvation stress response, a strategy thought to be common among native marine bacteria (29).

Recently, with increases in technology and the development of better microscopes with higher magnification capabilities, scientists have begun extending the lower limit for the possible presence of life. Many very small, bacteria-like structures, having a volume of approximately $8.2 \times 10^{-6} - 1.4 \times 10^{-2}$ μm^3, have been observed by SEM and TEM. Geologist R.L. Folk discovered these structures coined "nannobacteria," when studying travertine from the Le Zitelle Hot Springs of Viterbo, Italy (12). Nannobacteria are defined as small, spherical or rod shaped organisms ranging from $8.2 \times 10^{-6} - 1.4 \times 10^{-2}$ μm^3 in size. The smallest unit of nannobacteria noted as retaining a membrane has a

diameter of only 50 nm (2). Nannobacteria are found in clusters and in chains, characteristics of "normal" bacteria.

Since their discovery, nannobacteria have been noted in SEM/TEM images of samples from many places, including the San Marcos River (11) and tap water from The University of Texas at Austin (16), as well as mammalian blood and blood products (1,8,19,21). Researchers in Finland have discovered nannobacteria of sizes 2.7×10^{-4} – 6.5×10^{2} μm^{3} in both human and animal blood (19,21), and have tentatively named this novel bacterium *Nanobacterium sanguineum*. Ciftcioglu et al. have characterized the nannobacteria as having a high degree of resistance to heat, gamma irradiation, and antibiotics, although their multiplication can be prevented "with high doses of aminoglycoside antibiotics, EDTA, cytosine arabinoside and gamma-irradiation" (8), lethal conditions analogous to "normal" bacteria. It was also noted that the nannobacteria were able to form a biofilm containing hydroxyapatite.

Nannobacteria have been cultured in cell culture media, RPMI-1640 (Rockwell Park Memorial Institute-1640) and DMEM (Dulbecco's Modified Eagle's Medium) (9). Nannobacteria have been noted to have a negative effect on cell culture, ultimately resulting in vacuolization and cell lysis, possibly via receptor mediated endocytosis (7).

Research has implicated nannobacteria in the precipitation of minerals (13,38). If these are truly small forms of bacteria, it is not surprising that they could be associated with the formation of minerals. Much research has been done to document the link between normal-sized bacteria and mineral precipitation (27,33,34,38). The anionic cell surface is a major cause of bacterial enhanced mineral formation (28). Anionic carboxylate and phosphate residues on cell surface components such as peptidoglycan

(Pg), teichoic acids (TA), teichuronic acids (TUA) and lipopolysaccharide (LPS) impart an overall negative charge on the cell wall (28). Many bacteria have an outer surface referred to as a capsule, or glycocalyx, which may also contain anionic groups (such as the carboxylate or phosphate groups). Metal cations are attracted to this negatively charged area through electrostatic interactions. By-products of cellular metabolism such as SO_4^{2-} and S^{2-}, or subsequent changes in the pH surrounding the cell, also play a role in the precipitation of metals (28).

The most far-reaching significance of nannobacteria is their potential to exist in space or on other planets. The National Aeronautics and Space Administration (NASA) scientists have indicated the presence of these structures on the Martian meteorite ALH84001 (26), as well as on the Allende meteorite (16). If these structures are in fact biological, they could prove to be evidence for extraterrestrial life.

This research is important to many disciplines. If these purported organisms are playing a role in the formation of minerals, it is not entirely impossible that they may have played a role in the formation of rock structures present on Earth. These organisms may also play an unknown role in several disease processes. An example of this is the implication of aluminum in the development of kidney disease (25) and Alzheimer's disease (31). If aluminum is precipitated by nannobacteria, and nannobacteria can be found in blood, a logical step would be to determine what role these nannobacteria could be fulfilling in this process. To date, blood samples from patients suffering these types of illnesses have not been extensively examined with high power microscopy such as TEM or SEM. Another area that warrants research into the presence of nannobacteria is in the production of vaccines, some of which are produced using commercially provided

mammalian serum. Ciftcioglu and Kuronen et al. have isolated nannobacteria from more than 80% of commercial fetal and newborn bovine sera. Consequently, nannobacteria may truly be a threat to antibody products (8) that utilize sera in their production. If shown to exist, nannobacteria could be filling an ecological niche previously unexplored. This research attempts to answer the question of whether or not nannobacteria are real. A study of nannobacteria could also provide a link between the geological sciences and the biological sciences.

RESEARCH DESIGN AND METHODS

In order to address the question as to whether nannobacteria exist, several fundamental microbiological procedures were attempted. The initial step in investigating a potential new life form must be to find a suitable environment capable of supporting growth of the novel organism. In this research, many attempts were made to culture the organism from different sources in various environments. The possibility that the "organisms" are simply biological fragments was addressed through the addition of various enzymes to degrade any fragments from the samples. Morphological observations of the samples were made using two types of electron microscopy, TEM and SEM.

Samples

It has been proposed that nannobacteria play a direct role in the formation of minerals. A place where deposition of minerals (such as calcite and aragonite) occurs at a very high rate is in natural hot springs. For this reason, water samples were collected from the Jemez Springs in New Mexico and the Le Zitelle Hot Springs in Viterbo, Italy. Since nannobacteria are thought to be ubiquitous, water samples were also collected from the San Marcos River, as well as tap water from the Southwest Texas State University Science Building, in San Marcos, Texas for comparison. Tissue cultures from previous viral studies at SWT had been plagued with problematic growth that resulted in cell death. These tissue culture samples (chicken embryo cells-CEC), as well as several

commercial serum samples (fetal bovine and newborn calf; Sigma), were also screened for nannobacteria.

Isolation/Concentration

Attempts to isolate nannobacteria were made by filtering the water samples through a 0.45 μm Nalgene membrane filter followed by filtration through a 0.22 μm Nalgene membrane filter. This water was then ultracentrifuged at approximately 100,000 x g, at 4°C. A formvar-coated copper grid was placed at the bottom of the ultracentrifuge tube to allow for the collection of particles for visualization via electron microscopy. The supernatant was then poured off and another water sample (filtered as described above) ultracentrifuged in the same tube. This process was repeated three times to concentrate nannobacteria on the grid at the bottom of the tube. The concentrated nannobacteria were then examined by electron microscopy.

Culture

Several methods for culturing nannobacteria were performed. Traditional media types were inoculated with 0.1 ml each of concentrated sample described above: 1) R2A for the growth of oligotrophic organisms; 2) tryptic soy agar (TSA), a general nutrient media commonly used to culture a wide range of bacteria; 3) Sabouraud Dextrose Agar (SDA), a fungal-specific media; and 4) blood agar, a blood-enriched media. These plates were incubated at 25°C, 37°C, 37°C with CO_2 and 45°C until growth appeared. Samples from the Le Zitelle Hot Springs were incubated for one week at 45°C. Samples incubated at 45°C and 37°C were removed after two weeks to avoid dehydration of

media. All other samples were incubated for up to four weeks if no growth was noted. If growth occurred, the above process was repeated for the sample, with three replicates, to eliminate the possibility of the growth being the result of contamination. Cell culture media, DMEM and RPMI supplemented with 10% gamma irradiated fetal bovine serum, were also used. Both the DMEM and the RPMI were also prepared with the addition of 1% agar for solidification. DMEM is a chemically defined mixture of amino acids and vitamins, as well as additional supplementary components such as glucose, L-glutamine and sodium bicarbonate. These cultures were incubated at 37°C humidified, with 5% carbon dioxide, as well as at 37°C, 25°C, and 45°C without supplementation of CO_2 or humidity.

To eliminate the possibility of *Mycoplasma* being mistaken for nannobacteria, *Mycoplasma*-specific media, pleuro-pneumonia-like organism (PPLO) media supplemented with penicillin at 500 U/ml, was also inoculated with each sample at the time of inoculation of the R2A, TSA, blood agar, DMEM and RPMI media.

Dilutions of RPMI and newborn calf serum (0, 25, 50 and 75% serum) were performed and incubated in a humidified environment of 5% CO_2 at 37°C. Samples were checked for turbidity daily. Samples were also visualized via TEM weekly to monitor for growth.

Attempts were made to microculture colonies, as described by Torrella and Morita (35). A 2 cm x 2 cm square of an approximately 2 mm thick layer of agar (DMEM and R2A) was placed onto a clean glass slide. The agar had remained at room temperature for two days so that it would readily absorb a liquid sample. A 0.05 ml sample of CEC and tap water was filtered through a 0.22 μm filter, then placed onto the

agar square. A sterile coverslip was placed on top of the square of agar / sample. This sample was incubated inside a sterile petri dish on top of a piece of moist, sterile filter paper at 37°C. Slides were checked daily for growth using a phase contrast microscope.

Morphological Characterization

Samples of concentrated nannobacteria were gold coated using a 30 second coating-time, as recommended by Folk and Lynch (17), for visualization via scanning electron microscopy (SEM) at the University of Texas at Austin. Samples were also embedded in Spurr resin for thin sectioning and examination by TEM as described by Cheng et al. (6). Thin sectioning, which creates sections from 50 to 100 nm, allows for the visualization of internal cellular structures. Nannobacteria concentrated on the formvar-coated grids were negatively stained with 2% phosphotungstic acid (PTA). Negative staining allows for the visualization of external structures by TEM. Using this technique, samples were checked for the presence of external cellular structures such as a cell wall.

Three control grids were used for comparison. One grid was formvar-coated and stained with phosphotungstic acid (PTA), as all other samples had been. The second grid was formvar-coated, but was not stained with PTA. Finally, the third grid had a sample of sterile water mixed with control beads (0.11 μm polylatex microspheres, SPI #2709) centrifuged and stained with PTA in the same manner as previous samples. The control beads were used to ensure accurate measurement by TEM.

Composition Analysis

In order to determine if the structures seen were artifacts of biological molecules, DNAse, lipase, phospholipase and protease, were added the concentrated samples. Tap water samples were also mixed with Tween 20 (Sigma, P-1379), a detergent, then filtered through a 0.22 μm filter and ultracentrifuged onto a formvar coated grid as well. These samples were then viewed via TEM and SEM and compared to previous samples.

Samples of *Bacillus megaterium, Escherichia coli* and *Staphylococcus epidermidis* were inoculated into tryptic soy broth (TSB) and incubated at 37°C overnight. In sterile test tubes, 0.5 ml of each sample was mixed with 1.0 ml chloroform to lyse the cells. These samples were then filtered through a 0.22 μm filter and ultracentrifuged onto a formvar coated grid at 100,000 x g for 30 minutes. The grids were then negatively stained using 2% PTA for TEM. Due to mechanical failure, the *E. coli* samples were eliminated after ultracentrifugation.

Attempts were also made to visualize nannobacteria by fluorescent microscopy. Samples of water from the Jemez Springs, San Marcos River and SWT tap water were stained using 4',6'-diamidino-2-phenylindole·2 HCl (DAPI) stain (10 μl/ml, 20 minutes), which has an affinity for nucleic acids, then filtered onto a 0.22 μm polycarbonate filter. A control of *Bacillus megaterium* was made as well. The filters were placed onto glass slides with a drop of immersion oil, then viewed by epifluorescence microscopy.

RESULTS

Culturing nannobacteria. Attempts to culture nannobacteria on traditional culture media proved unsuccessful. The majority of plates did not show any growth throughout the incubation period (see Tables 1-5). A few plates did have colonies grow after a one-day period. The original water samples were re-plated, with three plates of the same media. These replicates were placed in the same environment as the original sample. In all cases, no growth was noted on the re-plating. The liquid media used (DMEM and RPMI) was equally unsuccessful in supporting growth of nannobacteria from all samples except the chicken embryo culture supernatant (see Tables 1-4).

Samples of water from the Le Zitelle Hot Springs in Viterbo, Italy also showed no growth when filtered through a 0.22 μm and a 0.10 μm filter. The control samples (not filtered) did not exhibit any growth on TSA or PPLO. There was abundant growth on the R2A by the control samples after one week of incubation at 45°C.

Microculturing attempts did not produce microscopically visible microcolonies throughout the three week period of incubation.

Scanning Electron Microscopy (SEM). Visualization with SEM revealed the presence of many spherical structures clustered together in the San Marcos tap water (see Figure 1a). Sizes of these potential nannobacterial structures range from 0.22 μm to 0.05 μm. Nannobacteria could not be visualized with SEM on the Jemez Springs samples, or the San Marcos River Samples. Many 0.1 μm spheres were evident in SEM pictures of the CEC supernatant (See Figure 2).

Transmission Electron Microscopy (TEM). Visualization of samples with TEM revealed nothing more than various irregularly shaped, globular-looking structures in all but the CEC and San Marcos tap water samples (See Figure 1b). Samples viewed by TEM of the CEC supernatant at 24 hours revealed the presence of many circular structures. These visible bodies appear to be surrounded by a membrane with a dense center. Sizes of these bodies range from 75 to 100 nm (See Figure 3). Images done at 96 hours reveal the presence of similar structures (Fig. 4). However, when images were shot at 5 days, these bacteria-like structures seemed to be decomposing, or losing their cellular characteristics. The membrane seems to have disappeared, and the structures appear more globular than cellular at this point (Fig. 5). Treatment of these samples (5-day old) with lipase, phospholipase, protease and DNAse did not significantly change the appearance of these globular structures, as illustrated by Figure 6.

Samples of water from the San Marcos River, Jemez Springs and Le Zitelle Hot Springs viewed by TEM also demonstrated various globular forms, but none with apparent cellular characteristics.

A control grid without sample, treated with PTA, was also observed. This image showed the presence of occasional groups of globular masses similar to the ones seen in many of the samples (See Figure 7). However, none of these masses appeared to have cellular characteristics. Another grid without sample, or PTA, did not show globular masses. The fields appeared free of microscopic debris. The final control grid, with the polylatex microspheres, shows the presence of the 0.11 μm control beads (See Figure 8).

Another image of the same beads illustrates the presence of a layer surrounding the beads, similar to that seen on bacterial cells (See Figure 9).

Attempts at preparing samples for thin sectioning were unsuccessful. The samples did not appear to absorb the osmium tetroxide.

Epifluorescent Microscopy. Nannobacteria could not be visualized using DAPI stain. Nannobacterial samples did not fluoresce as compared to the positively stained sample of *Bacillus megaterium* (See Figure 10).

Enzyme Experiment. Differences between samples with and without the addition of protease, lipase, phospholipase and DNAse were not noted (see Figure 11).

Sample: Tap Water

Media Type		25°C	37°C	37°C + CO_2	45°C
TSA	**a.**	a* NG	NG	NG	NG
	b.	NG	NG	NG	NG
R2A	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	NG
SDA	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	NG
Blood Agar		NG	NG	NG	NG
DMEM + agar		NG	NG	NG	NG
DMEM (liq)	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	NG
RPMI + agar		NG	NG	NG	NG
RPMI (liq)	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	NG
PPLO Agar		NG	NG	NG	NG

Table 1. Results of attempts to culture nannobacteria from SWT Science Building tap water on various media types. NG indicates that no growth was noted. (TSA=tryptic soy agar; SDA=Sabouraud's dextrose agar; DMEM=Dulbecco's Modified Eagle's Media ; PPLO=pleuro-pneumonia-like organism media, i.e. mycoplasma media) a* indicates growth on one plate, no growth on replating x 3.

Sample: San Marcos River Water

Media Type		25°C	37°C	37°C + CO_2	45°C
TSA	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	NG
R2A	**a.**	NG	NG	NG	NG
	b.	NG	a* NG	NG	NG
SDA	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	NG
Blood Agar		NG	NG	NG	NG
DMEM + agar		NG	NG	NG	NG
DMEM (liq)	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	NG
RPMI + agar		NG	NG	NG	NG
RPMI (liq)	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	NG
PPLO Agar		NG	NG	NG	NG

Table 2. Results of attempts to culture nannobacteria from the San Marcos River water on various media types. NG indicates that no growth was noted. (TSA=tryptic soy agar; SDA=Sabouraud's dextrose agar; DMEM=Dulbecco's Modified Eagle's Media ; PPLO=pleuro-pneumonia-like organism) a* indicates growth on one plate, no growth on replating x 3.

Sample: Jemez Springs Water

Media Type		25°C	37°C	37°C + CO_2	45°C
TSA	**a.**	NG	NG	a* NG	NG
	b.	NG	NG	NG	NG
R2A	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	a* NG
SDA	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	NG
Blood Agar		NG	NG	NG	NG
DMEM + agar		NG	NG	NG	NG
DMEM (liq)	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	NG
RPMI + agar		NG	NG	NG	NG
RPMI (liq)	**a.**	NG	NG	NG	NG
	b.	NG	NG	NG	NG
PPLO Agar		NG	NG	NG	NG

Table 3. Results of attempts to culture nannobacteria from Jemez Springs water on various media types. All samples were filtered through a 0.22μm filter. NG indicates that no growth was noted. (TSA=tryptic soy agar; SDA=Sabouraud's dextrose agar; DMEM=Dulbecco's Modified Eagle's Media ; PPLO=pleuro-pneumonia-like organism) a* indicates growth on one plate, with no growth on replating x 3.

Sample: Le Zitelle Hot Springs Water

Media Type	Sample	45°C, nonfiltered	45°C, 0.10μm	45°C, 0.22μm
TSA	Bh1d	NG	NG	NG
	An1d	NG	NG	NG
	Ab1d	NG	NG	NG
R2A	Bh1d	Abundant growth	NG	NG
	An1d	Abundant growth	NG	NG
	Ab1d	Abundant growth	NG	NG
PPLO Agar	Bh1d	NG	not tested	not tested
	An1d	NG		
	Ab1d	NG		

Table 4. Results of attempts to culture nannobacteria from Le Zitelle Hot Springs water on various media types. NG indicates that no growth was noted. (TSA=tryptic soy agar; PPLO=pleuro-pneumonia-like organism media)

Sample: Chicken Embryo Cell Culture Supernatant

Media Type	25°C	37°C	37°C + CO_2	45°C
TSA	NG	NG	NG	NG
R2A	NG	NG	NG	NG
SDA	NG	NG	NG	NG
Blood Agar	NG	NG	NG	NG
DMEM + agar	NG	NG	NG	NG
DMEM liquid	NG	NG	Growth noted	NG
RPMI + agar	NG	NG	NG	NG
RPMI liquid	NG	NG	NG	NG
PPLO Agar	NG	NG	NG	NG

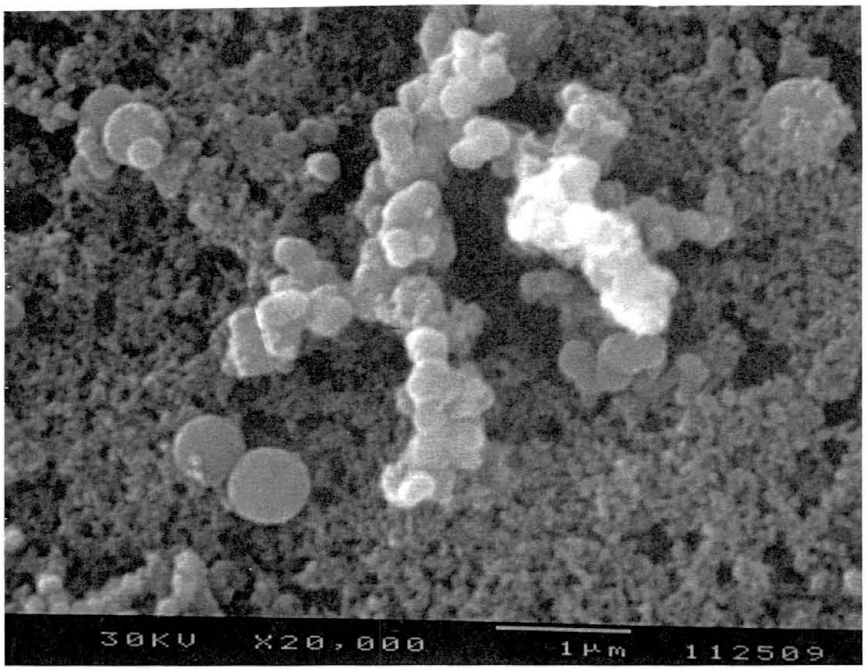
Table 5. Results of attempts to culture nannobacteria from Le Zitelle Hot Springs water on various media types. NG indicates that no growth was noted. (TSA=tryptic soy agar; SDA=Sabouraud's dextrose agar; DMEM=Dulbecco's Modified Eagle's Media ; PPLO=pleuro-pneumonia-like organism)

Serum Dilutions

Percent Serum		TSA	SDA	R2A
0%	A	NG	NG	NG
	B	NG	NG	NG
25%	A	NG	NG	NG
	B	**, NG	NG	NG
50%	A	NG	NG	NG
	B	NG	NG	NG
100%	A	NG	NG	NG
	B	NG	NG	NG

Table 6. Observations of plates made from samples of dilutions of newborn calf serum with RPMI. Samples were incubated in a humidified environment at 37C with 5% CO_2. NG indicates no growth noted after 2 weeks. **, NG indicates that growth occurred on the initial plate, but no growth occurred on the re-plating x 3.

a.



b.

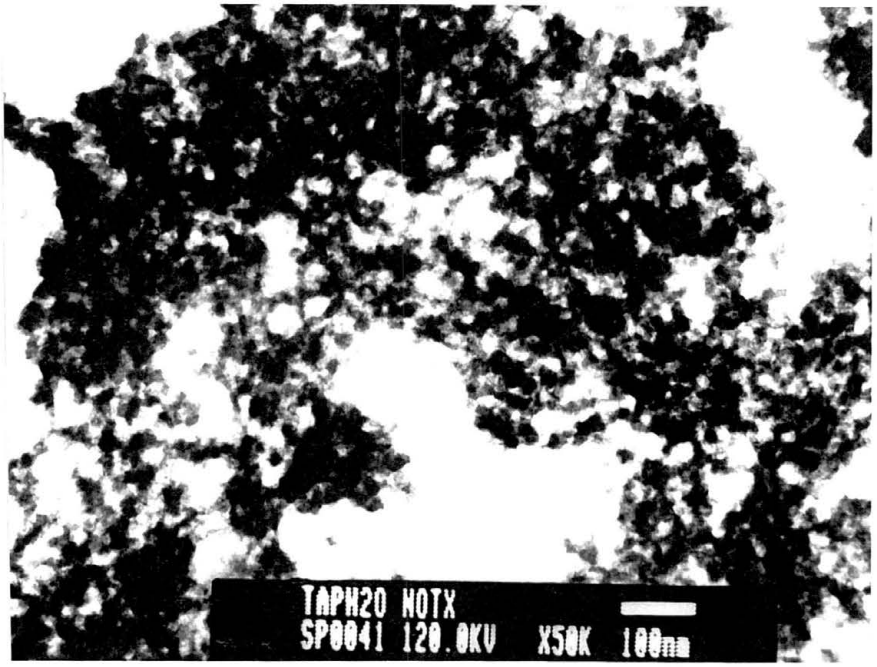


Fig. 1 Images of San Marcos tap water after filtration through 0.22 μm filter; **a.** SEM; **b.** TEM;

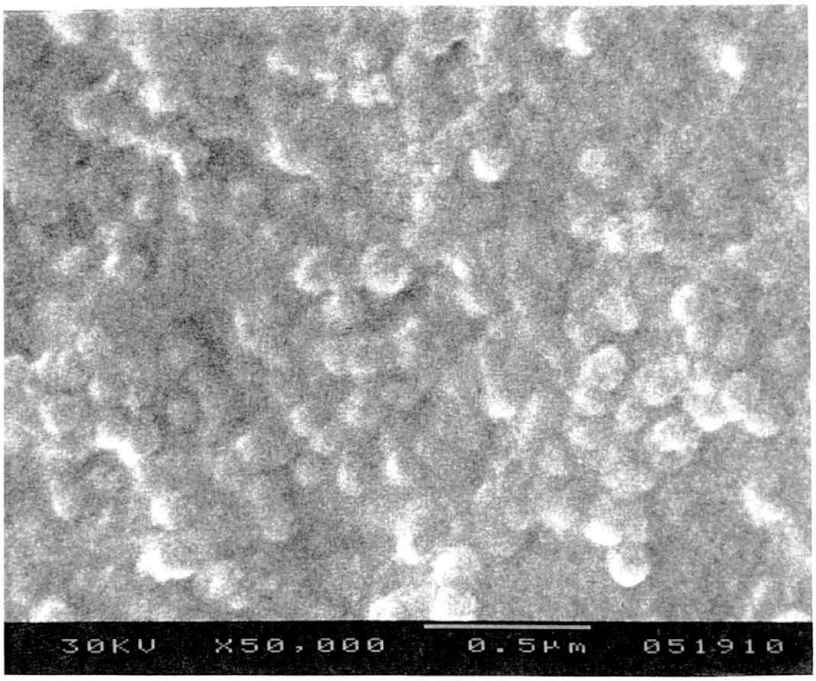


Fig. 2 Chicken embryo culture supernatant in DMEM at 24 hours, filtered through 0.22 µm filter, imaged with SEM.

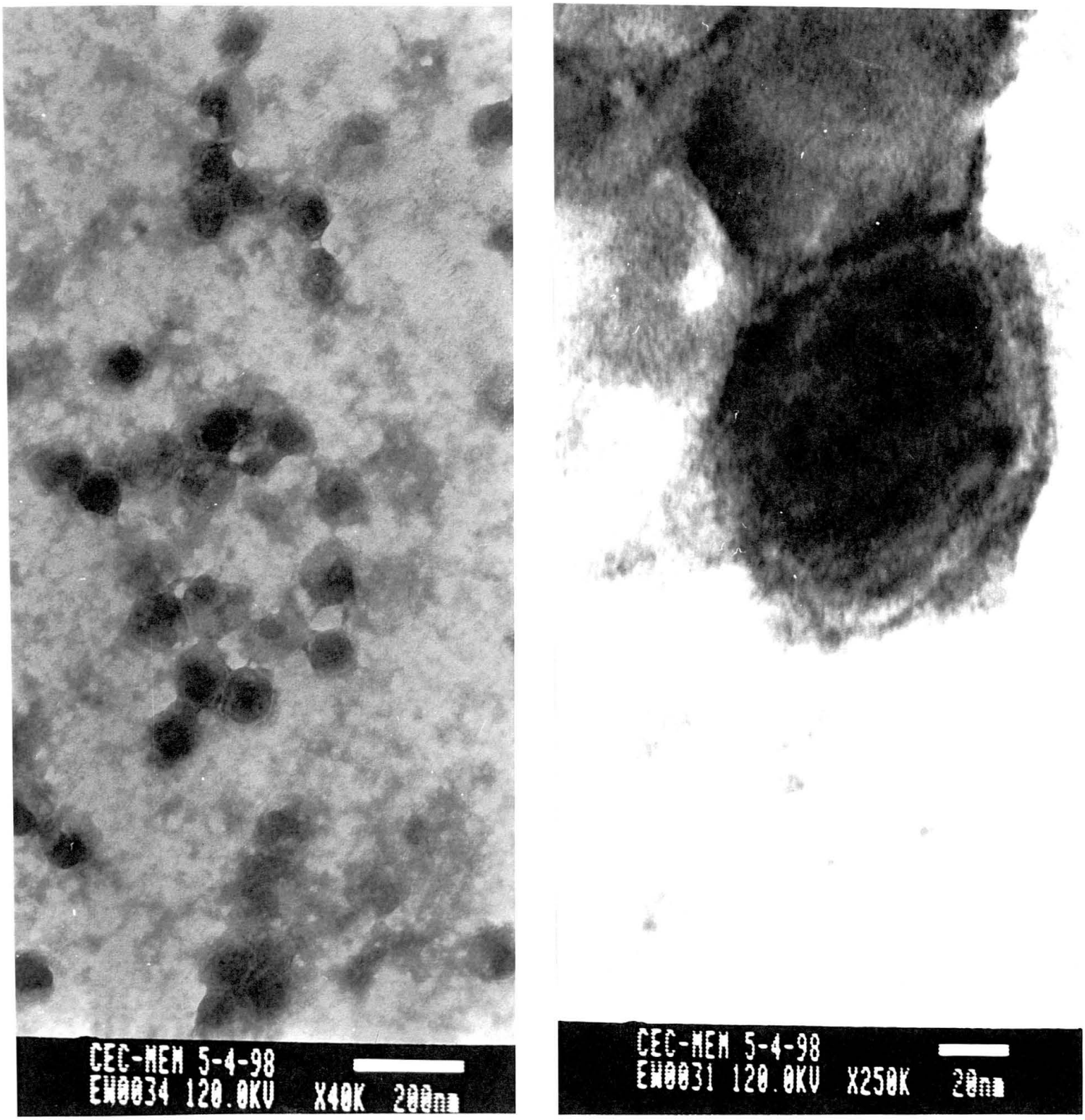


Fig. 3 Chicken embryo culture supernatant in DMEM at 24 hours, filtered through 0.22 μm filter, imaged with TEM; Image on the right is a close-up view.

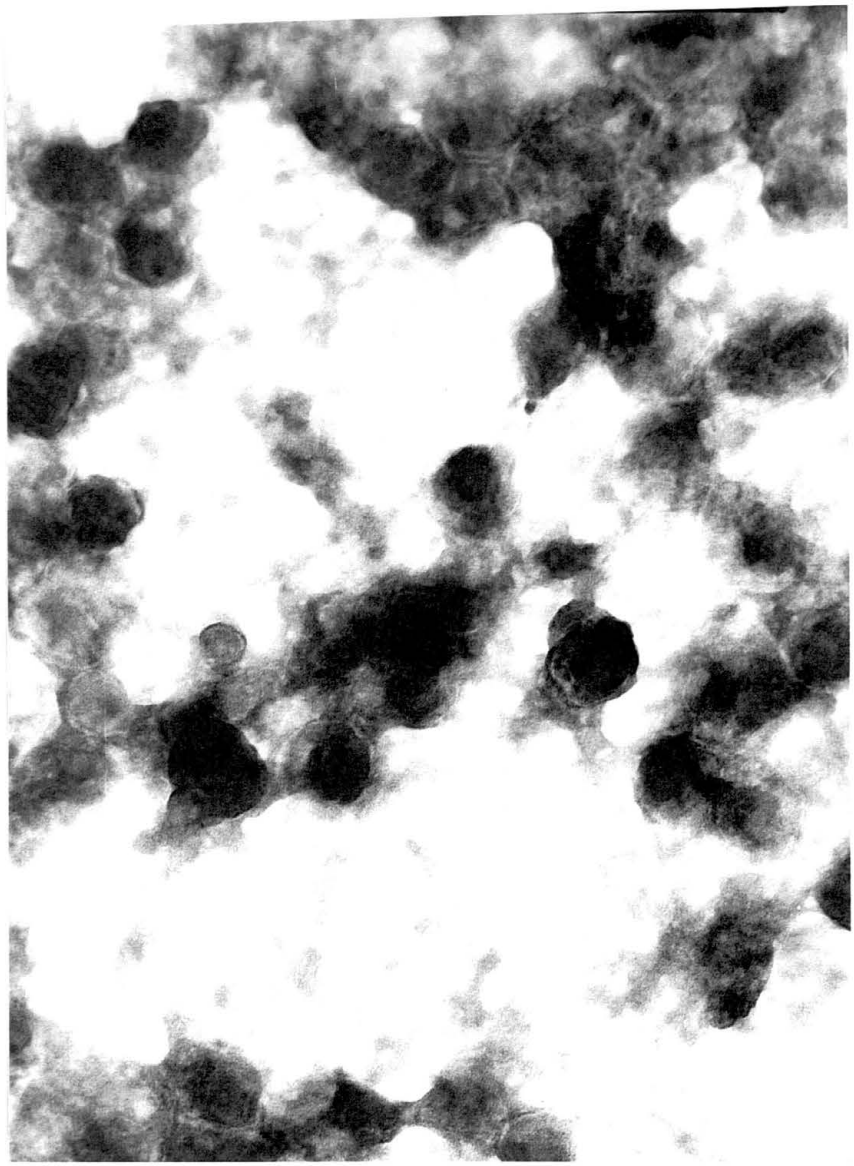


Fig. 4 Chicken embryo culture supernatant in DMEM at 96 hours, filtered through 0.22 μm filter, imaged with TEM.

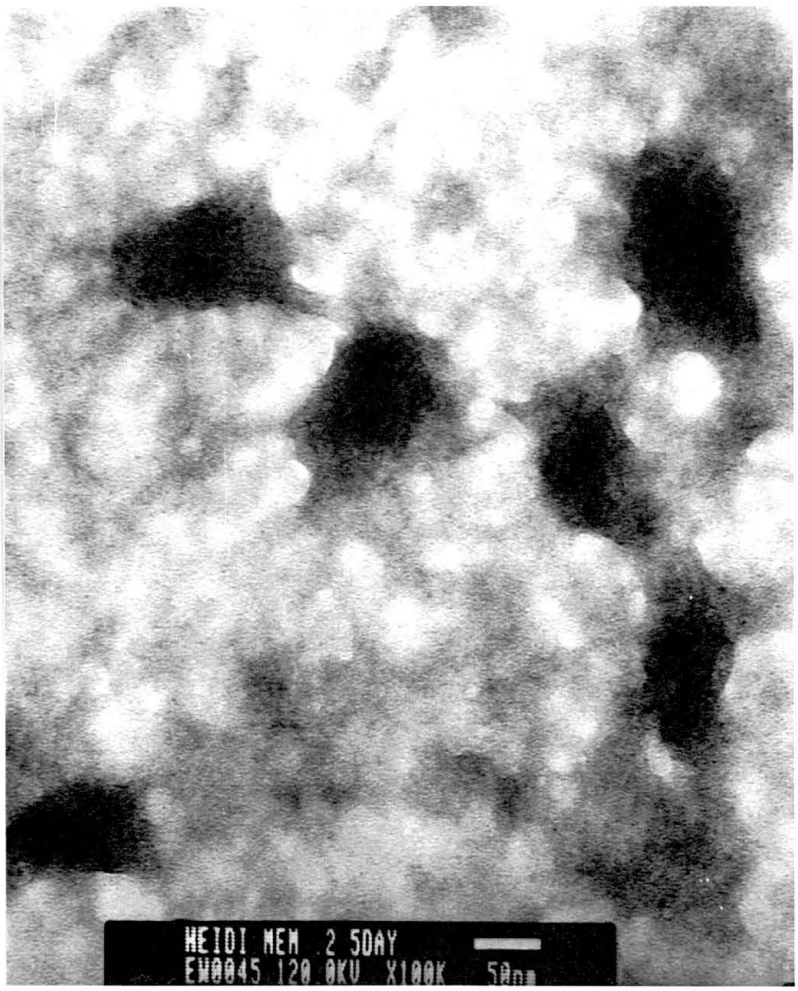


Fig. 5 Chicken embryo culture supernatant in DMEM at 120 hours, filtered through 0.22 μm filter, imaged with TEM.

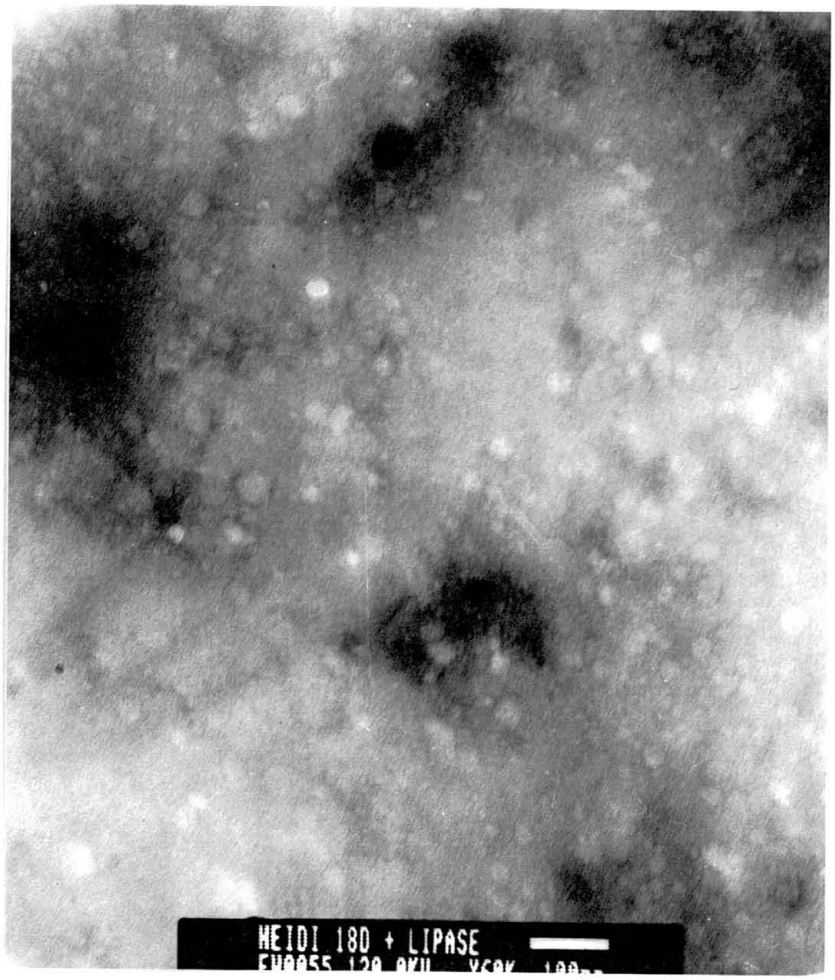


Fig. 6 Chicken embryo culture supernatant in DMEM at 120 hours, filtered through 0.22 μm filter, lipase added then imaged with TEM.

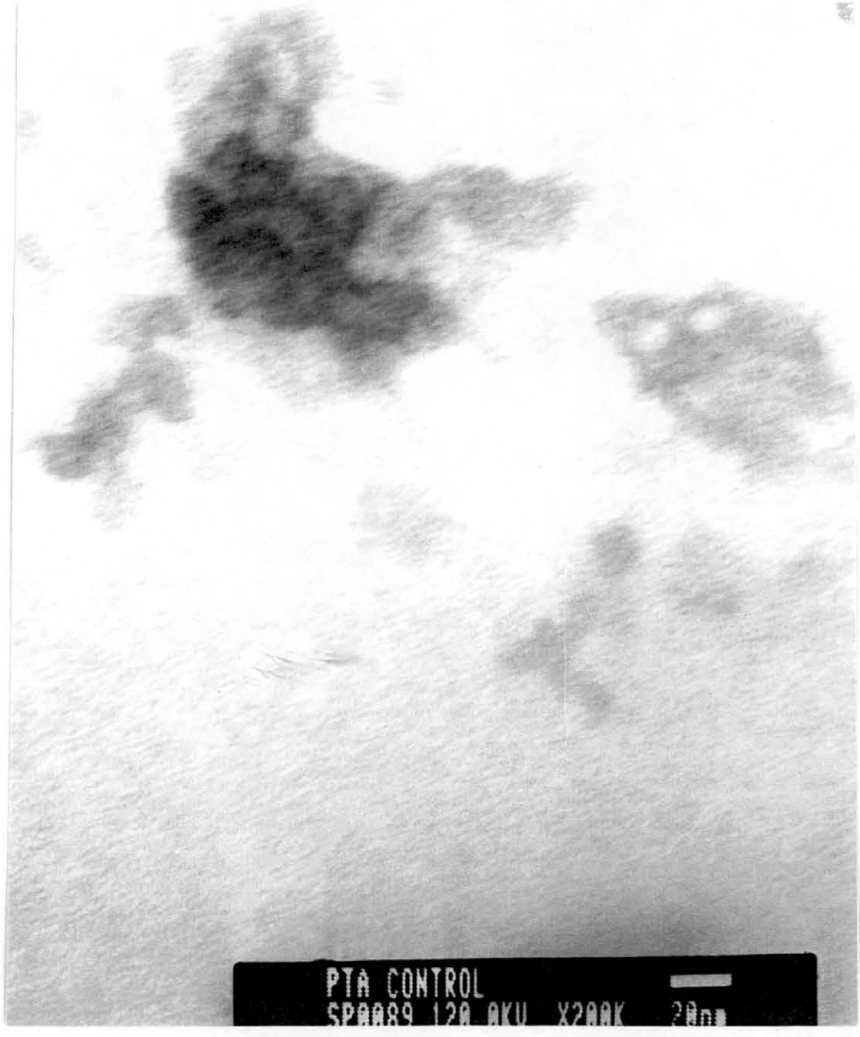


Fig. 7 Formvar coated Copper TEM Grid stained with PTA (no sample)

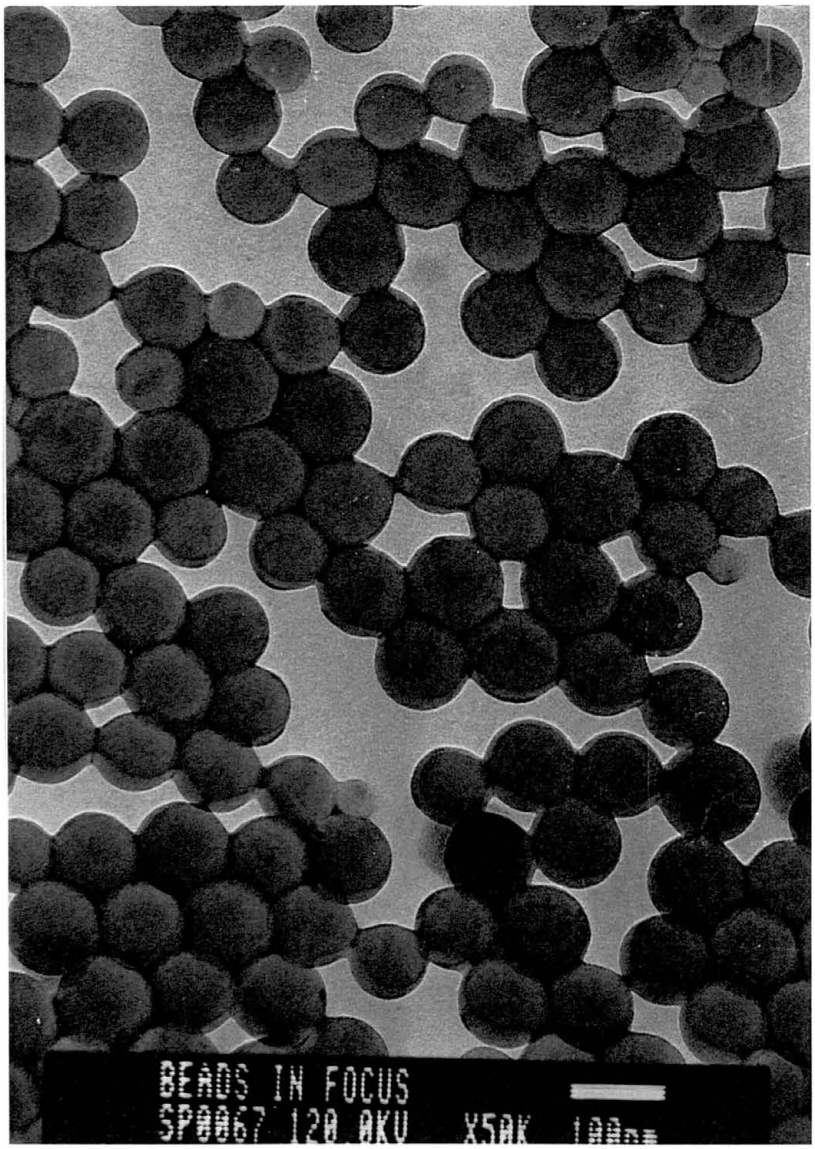


Fig. 8 Formvar coated copper TEM grid with 0.11 μm polylatex microspheres in focus.

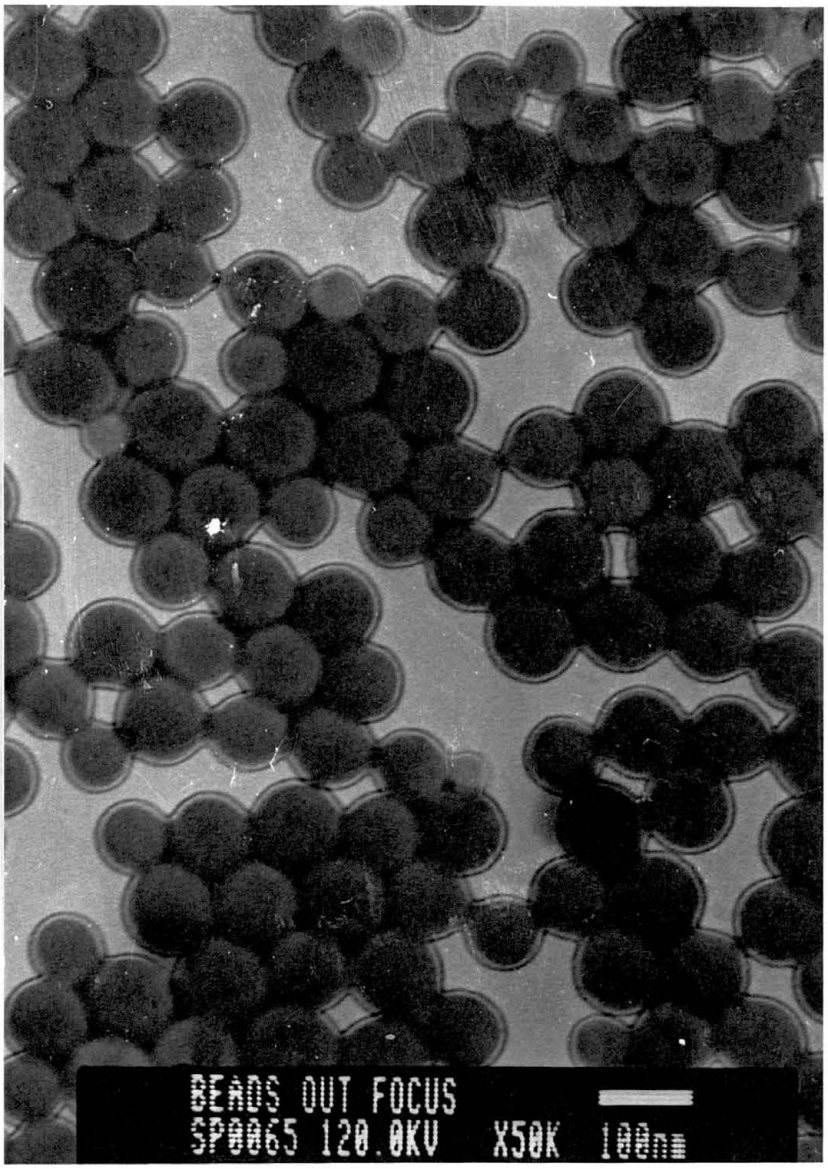
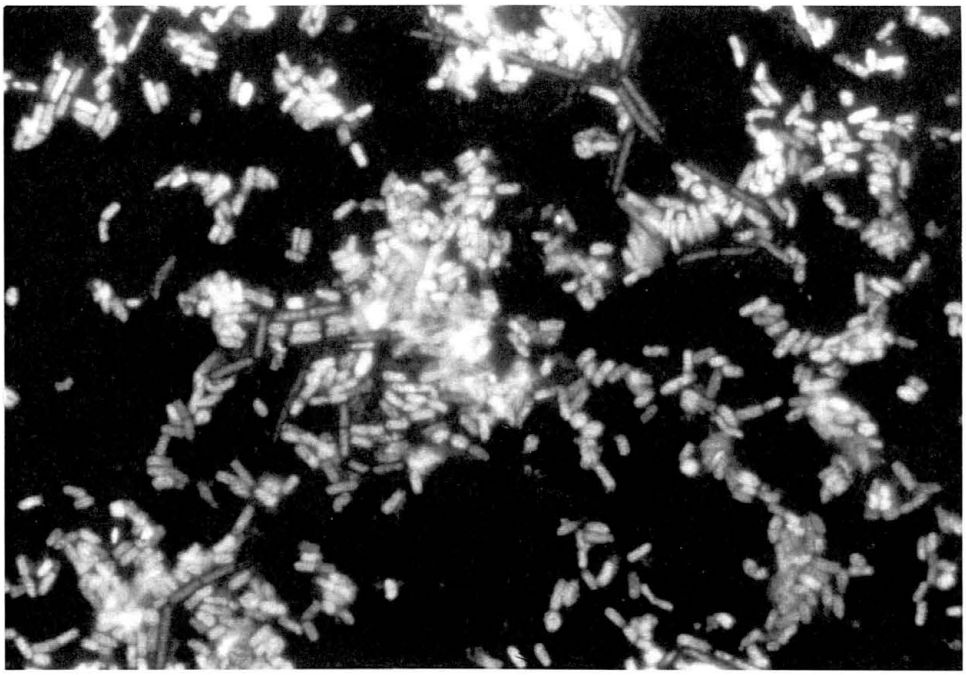


Fig. 9 Formvar coated copper TEM grid with 0.11 μm polylatex microspheres with focus slightly adjusted to create what appears to be membranes around the spheres.

a.



b.

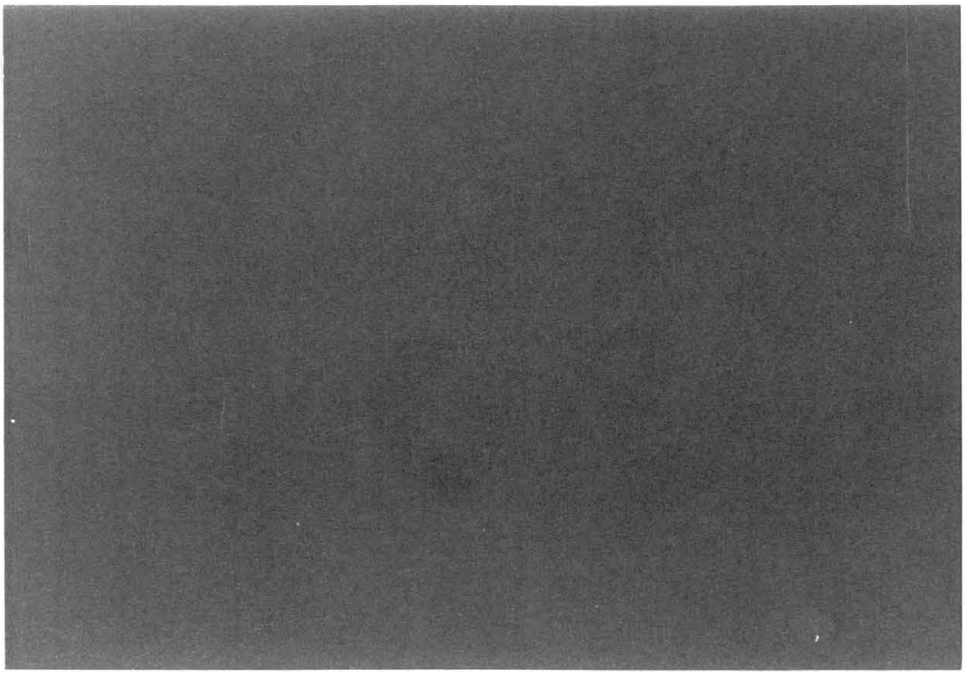


Fig. 10 a. Control *Bacillus megaterium* stained with DAPI and viewed with an epifluorescent microscope; b. Nannobacteria negative sample.

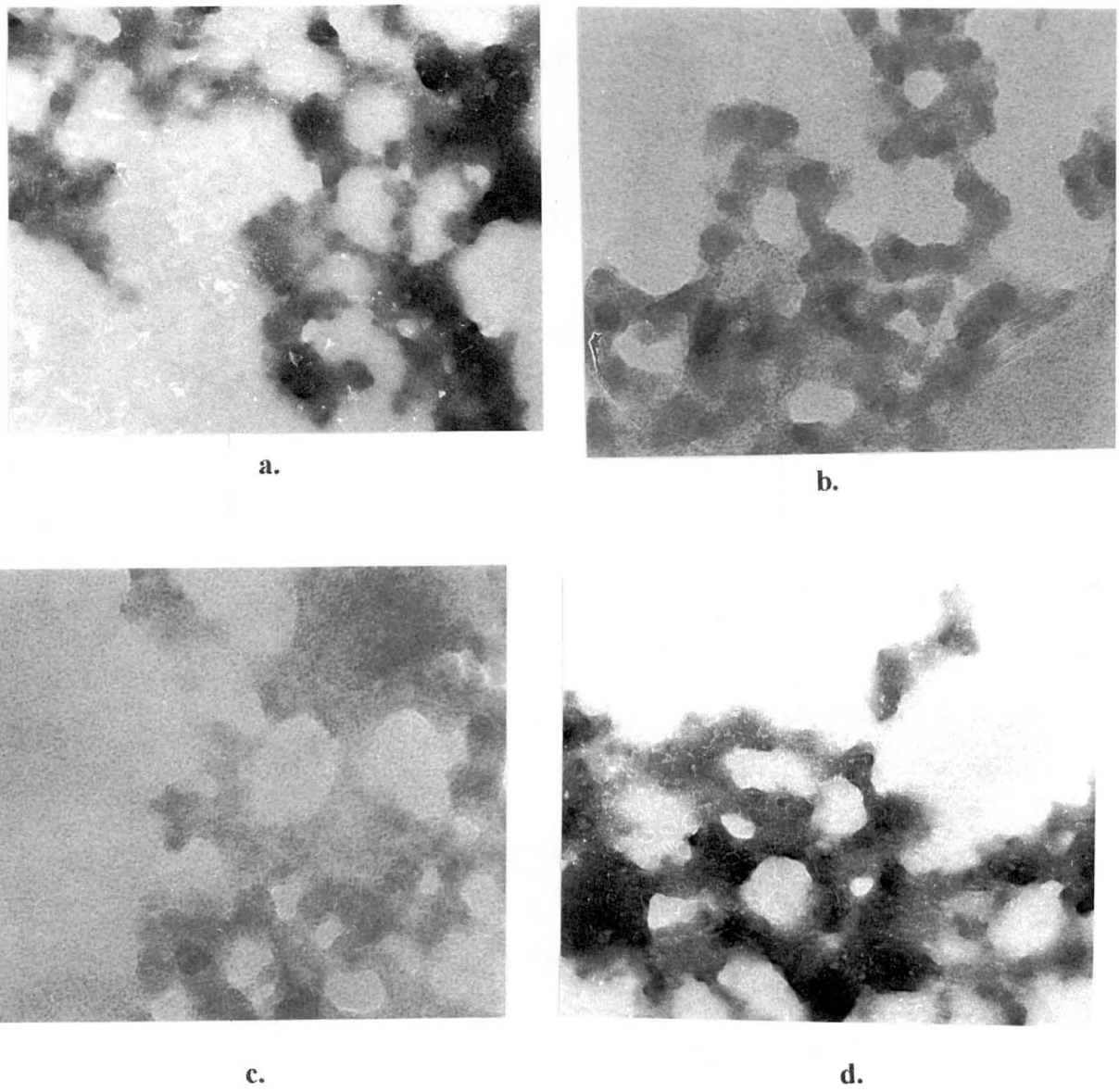


Fig. 11 Results of Enzyme Addition Experiment: 11a. tap water, no treatment; 11b. tap water + lipase; 11c. tap water + protease; 11d. tap water + DNAse; All images were made via TEM.

DISCUSSION

This discussion is divided into three sections. Section One will refer to Hypothesis #1, which states that nannobacteria are a new, independent life form. Section Two will be in reference to Hypothesis #2, which states that nannobacteria are biological fragments. Section Three will refer to Hypothesis #3, nannobacteria are non-biological artifacts.

Hypothesis #1: Nannobacteria are a newly recognized, independent life form. At the outset, I had hoped that if nannobacteria were truly a new, independent life form that it would be possible to culture them on some type of microbiological media. In this research, several media types were tested. Tryptic soy agar was used because it is a general nutrient media, commonly used for culturing bacteria. Sabouraud Dextrose Agar (SDA) was used to test for the presence of fungi in the samples. SDA is a nutritionally poor media with a pH of 5.6 (22). These conditions inhibit the growth of most bacteria while allowing the fungi to grow. Since fungal spores are approximately 1μm and fungal hyphae are significantly larger, it was not expected that samples filtered through 0.22 μm filters would show any growth. However, the potential for unusually small spores had to be eliminated. R2A is a media used to culture oligoheterotrophic organisms. Organisms normally found in water, such as the Jemez Springs, San Marcos River, Le Zitelle Hot Springs, and San Marcos tap water tested in this research, are usually culturable on this type of media because of its low nutrient content. The only growth on this type of media occurred when non-filtered control samples containing bacteria were plated on it. This provided evidence that the techniques employed for plating the samples could not be blamed for the absence of growth on the majority of the plates. The fact that only the

non-filtered samples grew into bacterial colonies suggests that the filtration techniques were successful in removing all traditional types / sizes of bacteria. Because nannobacterial researchers in Finland have stated that nannobacteria can be found in blood, blood agar plates were used with the hopes that it would create a suitable environment for the purported nannobacteria to grow. Cell culture media, DMEM and RPMI, were used in this research because Ciftcioglu et al. (19) have stated that these media types support the growth of nannobacteria. In the research described herein, samples filtered through 0.22 μm filters did not provide any growth on these media types. One other media type, PPLO media, used to isolate *Mycoplasma* species, was also employed in this research (PPLO stands for pleuro-pneumonia-like organism, a name given to *Mycoplasma* many years ago.). The media was supplemented with penicillin G to inhibit the growth of other bacteria types. *Mycoplasma* species are bacteria lacking a cell wall. The *Mycoplasmas* vary in shape from spherical (diameter 0.3 – 0.8 μm) to slender filaments (32). Because of their small cross-sectional diameter, it was thought that this might be seen in some electron microscopy and confused with nannobacteria. Since no growth was noted on the PPLO media at any temperature, *Mycoplasma* sp. can be eliminated.

Various environments were used throughout the isolation process. Samples were placed at 25°C, room temperature, 37°C, body temperature, 37°C with 5% carbon dioxide, and 45°C. These environments were chosen for several reasons. 37°C and 37°C with CO_2 were used because previous reports have stated that nannobacteria can be found in blood (body temperature), and successfully cultured at 37°C with CO_2 (7,20). Forty-five degrees was chosen so that thermophiles (organisms that grow best at high

temperatures) obtained from hot spring environments (temperatures ranging from 35.5-70°C; 13,18) could grow.

Samples of DMEM (liquid) did appear to support the growth of potential nannobacteria over a period of 4 days. The structures seen were spherical and surrounded by what appeared to be a membrane. Though this membrane structure could be a microscopy artifact, the consistency with which it appears suggests otherwise. After five days, the structures lost their cellular appearance. This could indicate that they are degrading in some way, possibly due to a change in the media composition through time. These results warrant further research.

Although this research was unsuccessful at culturing nannobacteria, it does not completely exclude the possibility of their existence. Due to the difficulty in creating an environment in which all organisms will readily grow, there are many organisms described as non-culturable. Originally, scientists had a difficult time proving that viruses existed when they failed to culture the organisms using traditional methods (24).

Hypothesis #2: Nannobacteria are biological artifacts. This hypothesis was tested through the enzyme addition experiments. It was thought that if the images seen were simply biological fragments, the addition of various enzymes (lipase, phospholipase, protease, and DNAse) would degrade the material, removing it from the field of vision in TEM. No differences were noted between the non-treated and the treated samples. This finding suggests that the particles seen are possibly non-biological, such as inorganic material or artifacts of microscopy.

Treatment of normal bacteria (*Bacillus megaterium*) with chloroform to lyse the cells did not create structures similar to those considered nannobacteria. In fact, the cells seemed to only shrink a bit with the addition of chloroform. This may be attributable to the fact that Gram positive organisms have a thick layer of peptidoglycan in their outermost layer. This may have protected the cells from complete lysis. Future tests of this sort should include the addition of a lysozyme as the initial step to avoid this problem, as well as the treatment of a Gram negative organism, which lacks the thick peptidoglycan layer seen in Gram positive organisms. In this research, a Gram negative organism, *E. coli*, was treated with chloroform. However, due to mechanical failure in the ultracentrifugation process, the sample was not imaged with TEM.

Some of the spherical structures seen could be a form of starved bacterial cells. It has been shown that normal bacteria, when placed in a nutrient-limiting environment, respond by a reduction in cell size (10). This reduction in size is thought to result in the production of ultramicrobacteria (29). Such ultramicrobacteria were described by Bae et al. (4) in 1972. Their research describes dwarf cells from soil, some of which were less than 0.08 μm in diameter, a size included in the description of nannobacteria!

Hypothesis #3: Nannobacteria are non-biological artifacts. There are artifacts inherent with all types of microscopy. TEM artifacts could be created through the process of staining with PTA. To rule out this potential, a control grid was imaged for comparison. There were a few globular structures seen (similar to some seen in the images of the water samples). This indicates that some of the structures seen in TEM can be created as artifacts of the microscopy process. A very interesting artifact

appeared in the TEM photos of the control grid with the 0.11μm polylatex control beads. It was found that by adjusting the focus slightly, a layer surrounding the beads would appear. This layer is seen clearly with the control beads (see Fig. 9). With random samples such as the water and serum samples, the potential for focusing-artifacts is just as high, but would be less obvious. Someone unfamiliar with this potential might claim to see a "cell membrane", erroneously considering a structure to be bacterial when in fact it is not.

Artifacts associated with the SEM may arise from many places. The first step involved with preparing samples for SEM is gold coating. It has been found that a gold-coating time of more than 30 seconds will create spherical shapes on the surface of a sample. These might mistakenly be called nannobacteria. Experiments at the University of Texas at Austin have concluded that a gold-coat time of less than 30 seconds will not create this type of artifact (14). Another potential artifact source associated with SEM is found in the etching process. Samples are often etched with an acid such as HCl. Current studies at UT Austin are investigating the possibility of creating nannobacteria-like spheres with various etching times and acid concentrations. Another source of potential artifact creation associated with SEM is based on the fact that the sample is placed in a high vacuum column. Research is warranted to determine the exact effect this type of environment is having on non-dehydrated biological samples.

Conclusion

This research has illustrated the inability to culture nannobacteria on several media types in various environments. However, these results do not exclude the

possibility of the existence of nannobacteria. At this point, I believe that there really is something present in the SEMs taken by researchers at the University of Texas (5,14,16), the University of Houston (5,15) and NASA (26). However, I do not believe that every tiny spherical shape visualized via high power microscopy is biological. We cannot and must not base conclusions on visual cues alone. Biological analysis should be used to determine the presence of biological characteristics such as reproduction, metabolic functions, etc. The determination as to whether or not something is biological cannot be based on its culturability alone. It is thought that as few as 2-4% of soil bacterial cells can be cultured (30)! It is also thought that some cells enter a state known as viable, but non-culturable (39).

Future work should include attempts to determine metabolic activity, using methods such as the measurement of the reduction of tetrazolium (37,40), as well as attempts to isolate genetic material from nannobacteria. PCR and genetic analysis could then be used to determine the fundamental relationship between normal bacteria and these purported organisms. All aspects of the production of nannobacteria-like features through artifactual creation must also be explored.

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