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MICROBIOLOGICAL ASSAY OF THE MSFC NEUTRAL BUOYANCY SIMULATOR

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16. ABSTRACT

Personnel safety for divers and astronauts, from the microbiological and medical viewpoint, is the primary reason for monitoring the Neutral Buoyancy Simulator water. Of secondary importance is the detection of microorganisms which may degrade the mockups in the water and the carpeted area around the simulator.

A Neutral Buoyancy Program was initiated in 1965 in the Process Engineering Laboratory, MSFC, to perform microbiological studies of the water in the 1.4-million gallon tank.

A broad spectrum of water sampling procedures was performed, and personal communications with mycology experts conducted to familiarize laboratory personnel with fungal problems in water.

With the addition of control procedures and remedial actions taken, the water was considered sanitary and in a safe condition for the divers and astronauts to perform their underwater neutral-buoyancy work.

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This document summarizes the microbiological investigations performed on the 1.4-million gallon Neutral Buoyancy Simulator from 1967 through 1972. The microbiologists who performed on the program were Mr. C. P. Shaia, Miss Diane F. Johnson and Dr. V. Grosse, Hayes International Corporation, with assistance of Messrs. T. Love, T. Lewis, and F. J. Beyerle, who provided the chemical analyses for this program.

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MICROBIOLOGICAL ASSAY OF THE MSFC NEUTRAL BUOYANCY SIMULATOR

INTRODUCTION

An 80-foot diameter by 40-foot deep Neutral Buoyancy Simulator (NBS) containing 1.4-million gallons of water is used to simulate a gravity-free state for evaluation of orbital operations. The water is maintained between 84° to 89° F or 29° to 32° C and is disinfected by maintaining a chlorine concentration of 1.5 parts per million (ppm) at a pH of 7.5 to 7.7. A pressure-type filter system with continuous slurry feed provides a complete turnover of the tank water in a 24-hour period.

Cleaning of the tank walls and normal maintenance of the tank structure is a time-consuming operation and is carried out as needed. Frequency of cleaning decreases in proportion to increasing water purity. Vacuuming of the tank floor, backwashing of the filter system and other cleaning operations are conducted on a regular upkeep basis.

The Neutral Buoyancy Program (NBP) was initiated in 1965 by converting a 55 000-gallon explosive forming tank into a NBS. Procedures for operating this small simulator to safeguard divers were obtained from literature and actual experience. In 1967 the large simulator was installed and professional microbiologists assisted in refinement of procedures for water safety control.

This report summarizes the microbiological investigations performed on the NBS from 1967 through 1972. It includes an ecological survey of the NBS to determine prevalent microorganisms. A second survey was conducted at a later date to determine if the prevalent microorganisms changed over a period of time. *Pseudomonas aeruginosa* and *Allesecheria boydii* were found in the simulator water. The simulator was drained and the inside surface was coated with Ceilcote 300. Tests were conducted prior to the coating that showed Ceilcote 300 was not a nutrient for microorganisms. After refilling, the simulator water was checked and found to be free of these two microorganisms, but the organisms were present in areas around the top of the simulator. A monitoring procedure was then established for routine checks in and around the simulator.

Additions of sodium citrate to the water were proposed as an inhibitor to oxidization of the aluminum parts in the simulator. Tests were conducted to determine nutrient characteristics of the sodium citrate in the water. It was found that the chemical was a growth-promoting factor for some microorganisms. Section VII gives representative results of the routine monitoring checks from July through October 1971.

In January 1972, the bottom samples were found to contain a granular debris. Microscopic examination showed many fragments of diatoms in the sediment. Cultures showed Allescheria boydii was present and was associated with diatomaceous earth debris at the bottom of the simulator. Section IX describes the study. The bottom of the simulator was vacuumed to remove the debris.

The microbiological work is described primarily in chronological sequence. Figure 1 shows a NBS for reference.

SECTION I. QUANTITATIVE BACTERIAL ASSAY AND ECOLOGICAL SURVEY OF THE NBS

On September 23, 1969, a fungus, Allescheria boydii, was detected, following procedures for routine water analysis of the simulator.

Fungi are microorganisms which contain no chlorophyll and must obtain food from dead organic matter or living hosts. Fungi distribution in nature is vast, and they can be found practically everywhere. Their range of activities include the rotting of soil, wood, and leaves to enrich the soil, expulsion of carbon dioxide into the air for utilization by green plants, and the production of organic acids for use in fermentation industries. Unchecked, the same fungi will cause deterioration of essential industrial and commercial materials and equipment, and disease in plants, animals, and humans.

The presence of a fungus in a body of water does not necessarily indicate the existence of a hazardous condition. The type of fungus, quantity present, the existing environmental conditions, as well as its natural distribution and clinical manifestations, must all be considered. For example, the fungus Allescheria boydii is a nonpathogenic inhabitant of natural waters and soils of this region. On the other hand, it has been isolated as a causative agent in Madura foot, a fungus disease occurring rarely in the United States.

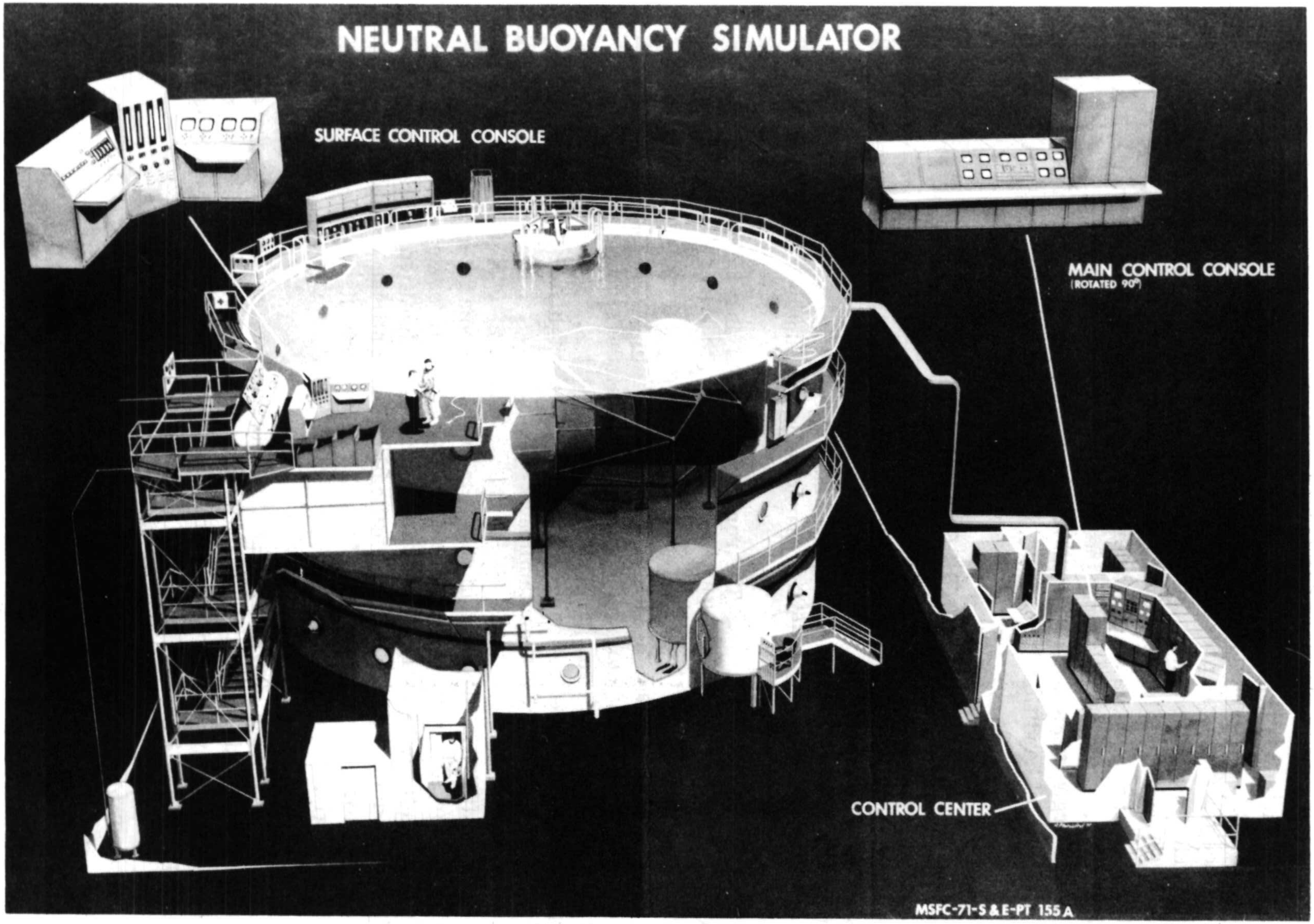

Figure 1. Neutral Buoyancy Simulator.

Between these extremes there exists a third condition: the occurrence of an organism, nonpathogenic in a natural state, in an artificial environment such as the NBS. Introduction of the organism into the system is relatively simple since the fungus is present in the surrounding air and soil. If the necessary conditions for growth exist in this artificial environment, the organism will establish itself. Without proper controls, the fungus will reproduce rapidly and thereby create a hazardous condition to equipment and man. The problem, then, is simply to supply the proper controls: (1) prompt recognition of the organism's presence, (2) determination of nutritional needs, and (3) elimination of conditions necessary for growth.

A. Laboratory Experiments

Allescheria boydii was discovered in a routine water-analysis sample. Intensive ecological surveys were conducted to obtain data on the growth requirements which the simulator environment provides, and community water sources were surveyed to furnish comparative data on natural contaminants.

1. Routine Bioassay. The routine microbial assay of the NBS was completed on August 28, 1969. The water sample yielded 0 organisms/ml after 48 hours incubation time, and a routine written report was issued. However, in order to eliminate all possibility of growth, the water-sample plates were retained in the incubator for several days after the standard incubation period. After 96 hours, a fungus appeared on the plates. Several procedures were initiated immediately to ensure that the fungus originated in the NBS and was not due to environmental contaminants.

After positive indication that the unknown contaminant originated in the simulator, the organism was sent to the medical laboratory in Huntsville for further identification. The fungus was tentatively identified as Allescheria boydii, a pathogenic fungus. This identification was confirmed by the State Health Laboratory in Montgomery and the Communicable Disease Center in Atlanta, Georgia.

2. Ecological Survey. A broad spectrum of water-sampling procedures was performed along with a literary survey, and personal communications with various mycology experts in the country so that laboratory personnel would be more familiar with the fungus.

To determine the extent of fungal contamination, the tank divers collected water samples from five selected areas within the tank on September

23, 1969. A viable organism count of 850 organisms/ml was recorded in one sample. Simulator officials and advisors decided to superchlorinate the simulator immediately. Chlorine concentration was held at 15 ppm for 96 hours.

Water samples were continued daily. Complete results of water sampling are contained in Table 1.

The literary survey of all available textbooks and related articles conducted by this laboratory, substantiated through personal communication with mycology experts, resulted in the following conclusions:

a. Allescheria boydii is an air and soil contaminant indigenous to this region.

b. Allescheria boydii can utilize a wood or rubber substrate for growth in an artificial environment.

c. It is likely that a bacterial scum adheres to the sides of the simulator and that various microbial niches exist in the crevices and cracks of the simulator and the mockups which might supply the nutritional needs of the fungus.

d. The organism appears to be extremely resistant to chlorine. It is possible that this particular organism is capable of incorporating the chlorine as a nutrient adjunct.

e. Present chlorination and filtration systems of the NBS appear adequate under normal conditions. However, special procedures are in order to eliminate present gross contamination. Additional preventive measures must be incorporated in the routine upkeep of the simulator by both the simulator officials and this laboratory.

3. Comprehensive Sampling. A comprehensive sampling of the NBS on October 20, 1969, provided the data discussed in this section.

a. Materials. The materials used were Sabouraud maltose agar plates (Sab malt), sterile sampling equipment, sterile culture equipment, incubator, and PathoTec (differential test papers).

b. Procedures. The following tests were performed in collaboration with a mycology professor at Georgia State University.

TABLE 1. QUANTITATIVE MICROBIOLOGICAL ASSAY OF NEUTRAL BUOYANCY SIMULATOR WATER

Prior to Superchlorination		
Date	Location	No. Organisms*/liter
9-8	spigot	10
9-15	spigot	20
9-26	workshop	100
9-26	forward dome	850
9-26	airlock	130
9-26	diving bell	230
9-26	outlet to spigot	110
After Superchlorination		
10-6	spigot	1
10-7	spigot	22
10-8	workshop	1
10-8	forward dome	13
10-8	airlock	2
10-8	diving bell	0
10-8	spigot outlet	2
10-9	spigot	10
10-13	spigot	70
10-14	spigot	60
10-15	forward dome	100
10-16	spigot	30
10-17	spigot	10
10-20	spigot	0
10-21	spigot	0

* "Organisms" in this case are *Allescheria boydii*.

The NBS and surrounding area were examined for possible substrates favorable to the environmental needs of the organism, Allescheria boydii. Twenty sites were selected and subsequently samples were taken. A variety of sampling techniques was utilized, including water collections, material samples and surface scrapings. The sites chosen and methods utilized are illustrated in Table 2.

Duplicate samples of each site were cultured. Georgia State University used a mycological agar with special additives (Myco C-L). This is a neutral medium recommended for fungi requiring neutral or slightly alkaline medium. This laboratory used Sab malt agar, which is particularly effective in the cultivation of Allescheria boydii and Pseudomonas.

All samples cultured on Sab malt were incubated at 37°C for 96 hours.

c. Results. Various species of fungi were recovered from twelve samples: Allescheria boydii, Fusarium species, Alternaria species, Aspergillus species, Penicillium species, and Rhizopus species.

The organism Pseudomonas species was identified from three samples. This identification was based on visual observation of the organism's characteristic growth on Sab malt agar, microscopic examination and differential testing with PathoTec.

In addition, a gram-negative bacterial growth other than Pseudomonas was observed in five samples.

A Streptomycetes species was identified from a gross air-sampling procedure.

Dr. Ahearn, of Georgia State University, analyzed and identified all fungal cultures from both laboratories. The data illustrated in Table 2 represent the combined results of both labs.

4. Discussion. The results of the tests reinforced certain theories concerning contamination of the NBS. The existence of a bacterial scum and the occurrence of a rubber or wood substrate were originally indicated by this laboratory as the most probable essentials for growth of a fungus in the NBS. Under the direction of Dr. Don Ahearn, the necessary measures were performed to validate these theories.

TABLE 2. RESULTS OF ECOLOGICAL SURVEY OF NEUTRAL BUOYANCY SIMULATOR

Sample Number	Source of Sample	Method of Sampling	Organisms Identified
1	rubber coating on ladder	material sample	light bacterial growth *Allescheria boydii*
2	roofing	surface scraping	no growth
3	tear in roof	surface scraping	no growth
4	inside forward dome	surface scraping water collections	*Allescheria boydii*
5	white precipitate on mockup	material sample water collections	*Allescheria boydii*
6	rubber/mat bottom	material sample	*Allescheria boydii* *Fusarium* bacterial growth
7	ports	water collections	*Allescheria boydii*
8	vacuum hose	material sample	*Allescheria boydii*
9	floor - under carpet	surface scraping	*Fusarium*, *Aspergillus*, *Penicillium* species abundant bacterial growth
10	diver's suit	surface scraping	no growth
11	diving bell	water collection surface scraping	abundant *Pseudomonas* species growth
12	water sample - outgoing spigot	water collection	no growth

TABLE 2. (Concluded)

Sample Number	Source of Sample	Method of Sampling	Organisms Identified
13	water sample - ingoing spigot	water collection	no growth
14	concrete blocks (latex paint)	material sample	<u>Allescheria boydii</u>
15	styrofoam	material sample	bacterial growth <u>Pseudomonas</u> species <u>Allescheria boydii</u> <u>Rhizopus</u>
16	rubber strip (tank bottom)	material sample	bacterial growth <u>Fusarium</u> species
17	sponge rubber (tank bottom)	material sample	bacterial growth <u>Pseudomonas</u> species <u>Fusarium</u> species
18	hose	surface scraping	<u>Allescheria boydii</u> <u>Alternaria</u> species
19	scum (side of tank)	cotton swab	no growth
20	scum (diver's side of tank)	cotton swab	no growth
21	air	fallout	<u>Streptomycetes</u> species

As the results indicated, a fungus was growing on the various rubber constituents in the simulator. In addition, a bacterial scum was recovered by scraping various surfaces.

An interpretation of these results also indicated that the primary source of growth was not in the air or air surfaces. However, it was most probable that the initial source of contamination by Allescheria boydii was through airborne contaminants.

A second major fungal contaminant, Fusarium, was found growing in an almost pure culture on the bottom of the indoor-outdoor carpeting. Fusarium is a slightly-pathogenic fungus whose numbers should be controlled. It also thrives on rubber or rubberized substrates and was probably introduced by air or from dirt tracked in by personnel.

All fungal and bacterial contaminants are indigenous to the area and may be expected to occur occasionally. They will not thrive in epidemic numbers unless the proper substrates are provided for growth.

5. Field Survey. A field study of local waters and their natural contaminants was conducted. This was done to verify the theory that common contaminants of the NBS were indigenous to the area. The survey was very basic, including only water sampling with no air and soil samples taken.

a. Materials.

(1) Nutrient media

(a) Blood agar
(b) Trypticase soy agar
(c) MacConkey's agar

(2) Differential media

(3) Biochemical assay media

(4) Sterile collection equipment

(5) Culture-handling equipment

b. Procedures. The samples were taken at the locations described in Table 3. All samples were taken on a clear, cool day with little

TABLE 3. RESULTS OF FIELD SURVEY

Sample Number	Source of Sample	Colony Count Organism/liter	Major Organisms Identified
1	creek in city	TNTC*	Proteus vulgaris Sphaerotilus natans Arthrobacter globiformis Spirillum undula yeast species
2	large, man-made lake	3000	Flavobacterium arborescens Micrococcus species Thiobacillus species Fusarium species
3	large, outdoor public pool	500	Flavobacterium arborescens gram-negative bacteria
4	river	TNTC*	Pseudomonas species Flavobacterium arborescens Gallionella species (tentative) yeast species Fusarium
5	creek in rural area	800	Escheria coli Sphaerotilus natans Pseudomonas species Thiobacillus species Arthrobacter globiformis
6	small indoor public pool	0	No organisms recovered

* Too numerous to count

wind. The outdoor pool was closed for the season, and the indoor pool had not been utilized on the sampling day. The sites were selected to give a representative picture of local water microbial flora.

Organisms were recovered from the water in two ways. Quantitative data were obtained by filtration of 100-ml water from each sample. Five-ml aliquots of the original samples were taken for pour plate qualitation.

All plates were incubated for 72 hours at 36°C.

c. Results. A large variety of organisms was recovered from all samples except from the indoor pool, as shown in Table 3. The natural contaminants identified included Fusarium and Pseudomonas. A more intensive sampling would probably have produced the entire range of fungi found in the NBS.

Comparison of the natural waters with the biocontamination of the NBS reveals that this "pool" was comparatively clean. It must be noted however, that natural waters were not used for valuable mockups and neutral buoyancy simulation. Therefore, excessive emphasis could not be placed on the relative "cleanness" of the NBS.

B. Recommendations

Primary emphasis thus far has centered on the detection of organisms in the artificial environment and recognition of their nutritional growth requirements. The remainder of this section is concerned with the third phase of proper control, the elimination of conditions necessary for growth. Efficient methods of microbial control are based on the subjection of organisms to an injurious chemical substance or an unfavorable physical condition. However, microorganisms vary in their susceptibility to physical and chemical agents. The intensity and nature of the chemical and physical agents, the type of microorganism involved, as well as the physical and chemical properties of the substance bearing the organisms have an important influence on the rate and efficiency of microbial destruction. There is no single antimicrobial agent "best" for all purposes.

Various methods of decontamination of the NBS were suggested. These are included with a brief mention of their relative effectiveness or ineffectiveness. The recommended measures adopted by this laboratory were based on the data previously presented. The proposed method for immediate decontamination includes an outline of preventive measures.

The choice of agents for microbial control were limited by three factors: (1) toxicity to man, (2) corrosive effects on materials, and (3) availability.

1. <u>Proposed Decontamination Methods.</u>

a. Weak Organic Acids (Calcium or Sodium Propionate). These weakly-oxidizing agents might prove effective for some of the pool contaminants. However, selection of the proper acid, determination of dilution factor, and broad spectrum in vitro tests to determine organism susceptibility would require an excessive amount of time.

b. Copper Sulfate. This chemical is effective against algae, but no data are available on the effectiveness against fungi. Since organisms were not growing primarily in a wall scum which could be based on algae, elimination of algae would not affect growth requirements for the contaminant fungi. In addition, copper sulfate may be toxic in concentrations greater than two parts per million. The compound is precipitated by alkaline carbonate and rendered ineffective. Also, it will discolor swimmers' hair and suits.

c. Chlorine. This chemical is probably one of the most effective decontamination measures for any pool. However, chlorine is most active against organisms in the free water. Most microorganisms survive and reproduce in organic scum along walls and equipment, as well as in any cracked or abraded surfaces. Chlorine cannot penetrate these microbial niches; therefore, chlorination must be accompanied by good housekeeping techniques.

d. Iodine. This chemical is equal to chlorine as a decontaminant and possesses added advantages, as it is less irritating to the eyes and skin of divers. Chlorine is somewhat easier to stabilize in a system. Alternative use of chlorine and iodine might eliminate the development of organisms resistant to a continuously-used control agent.

e. Heavy Metal Compounds and Ions (Mercuric Chlorides, Colloidal Silver Compounds, Ag, Hg, Fe). Unless such compounds are used in a paint base to decrease buildup of organic scum, their toxicity and potential corrosiveness make them unsuitable candidates for pool decontamination.

f. Thermal Decontamination. It was suggested that steam could be added to maintain the water temperature at 55° to 60°C for 1 hour. The size and volume of the simulator make this method impractical.

g. Ultraviolet Light. This is not a recommended method for decontamination of pools.

2. Recommended Decontamination Methods.

a. Remove indoor-outdoor carpet. Replace with a gridded non-slip surface material.

b. Remove all rubber and polyethylene substrates from simulator.

c. Scrub simulator walls regularly to remove bacterial scum and to prevent buildup of microbial niches. It may be necessary to drain simulator and wash the bottom and sides with a 5-percent hypochlorite solution.

d. Repair cracks and material abrasions. Create as nearly as possible a smooth, durable surface.

e. Investigate the possibility of coating surfaces with fungicidal paint.

f. Require divers to shower with soap before entering pool. Use foot powder to combat athlete's foot.

g. Disinfect simulator suits and diving equipment with Zephiran solution or ultraviolet light.

h. The temperature of the simulator water is 10° F (3° C) higher, 84° to 89° F (29° to 32° C), than the standard heat requirement of indoor swimming pools, 74° to 79° F (23° to 26° C). This elevated temperature provides excellent incubator conditions.

i. Allow no street shoes on upper deck of simulator. Despite the public-relations problem inherent with this suggestion, the problem of biocontamination makes the proposal an essential item.

j. A biannual ecological survey of the NBS should be conducted by this laboratory to maintain a close check on the microbial load. Newer techniques will be incorporated in the monthly routine bioassay.

C. Conclusions

The heavy bioload of the NBS was not a panic situation, and adequate controls should have been recognized and used. The organisms detected in the simulator were indigenous to this area. They were natural inhabitants of the water, soil, and air. Fungi spores were transported from their natural environments to the NBS via air convections, material contaminants, and human carriers. In addition, each swimmer that entered the simulator introduced his own normal microbial flora. If the proper conditions for growth existed, these various organisms continued to live and reproduce. For example, Allescheria boydii and Fusarium utilized a rubber or wood substrate for their life requirements. Both materials were located within the simulator system. Hence, a life cycle was established.

A chlorine control system will effectively monitor microorganism contamination, unless the organisms have developed an adequate protection system which prevents penetration of the chemical or have produced chlorine-resistant mutations. Human mucoid and oil secretions, chemical precipitation, irregular water-circulation, and abraded surfaces lend themselves to the formation of a protective bacterial scum.

To effectively monitor the contaminant buildup in the NBS, it is necessary (1) to remove the material substrates, (2) to maintain a smooth durable surface within the tank, (3) to provide a means for routine removal of bacterial scum on the simulator sides, and (4) to reduce the microbial influx.

If these simple techniques are followed, the viable organism count of the NBS will be maintained at a safe minimum, and personnel and equipment will be protected.

SECTION II. ECOLOGICAL SURVEY OF THE NEUTRAL BUOYANCY SIMULATOR

The ecological survey of the NBS was repeated from June 1 to July 6, 1970, to identify those aerobic, sporogenous and asporogenous heterotropic mesophiles which were most prevalent in the system at this time. Most of those organisms reported to cause pathogenic conditions in persons frequently in contact with swimming-pool water can be classified in the above-named group. Anaerobic and fastidious organisms requiring special media were not included in this study because of the time allotted and because this group of organisms was expected to comprise only a small portion of the flora of the air, water and carpet.

The data collected from this study will help direct efforts toward decreasing the total contamination level on the upper deck of the NBS and eliminate conditions which may give rise to the harboring of potential pathogenic microorganisms.

A. Materials

The following culture media, apparatus, and reagents were used to perform this study:

1. Reyniers slit air sampler.
2. 150 × 15 ml TSA plates (plastic, sterile).
3. Sterile 100-ml glass bottles.
4. Molten TSA.
5. 100 × 15-ml TSA plates.
6. Lactose broth.
7. Sodium thiosulfate, 0.02 gram/100-ml bottle.
8. Blood agar plates.
9. Sabouraud-dextrose slants and plates.
10. Rodac plates (TSA, EMB, SAB).
11. EMB plates.
12. 11-mm diameter borer.
13. Blender.
14. Peptone water, 0.25 percent.
15. Lacto-phenol cotton blue.

B. Methods

1. Air Sampling. Two samples were taken on 150- × 15-mm TSA plates of the air around the instrument area of the NBS using the Reynier slit-air sampler. The samples were taken on different days, and the air was sampled for 1 hour at the rate of 1 ft^3/min. After sampling, the plates were incubated for 24 hours at 35° C. Total colony counts were made and the organisms identified [1].

2. Water. Three sets of water samples were taken on different days. The first set of water samples was collected in 100-ml sterile bottles containing 0.02 grams of sodium thiosulfate evaporated in the drying oven. The sodium thiosulfate neutralized the inhibitory effect of the chlorinated water on microorganisms. Samples were taken from the NBS surface, over a flow basin, bottom, potable water line, television camera, and metal slugs at the bottom of the simulator. TSA plates were made in duplicate using 1-ml and 0.1-ml aliquots of each sample. These plates were incubated at 35° C for 96 hours. After incubation, the colonies were counted and the organisms identified. In addition, 15 ml of double strength lactose broth was innoculated with 15 ml of each sample and checked for acid or gas formation after incubation at 35° C for 48 hours.

The second set of specimens consisted of four samples, two from the surface of the simulator and two from the bottom. One sample at each location was taken in a sterile bottle containing 0.02-gram sodium thiosulfate, and one sample from each location in a sterile bottle containing no sodium thiosulfate. TSA pour-plates were prepared in duplicate from these samples, as described in the above paragraph. To determine the inhibitory effect in the second set of samples of the chlorine in the water and the effectiveness of sodium thiosulfate in neutralizing the chlorine, control samples were run on each of the four samples by inoculating aliquots of each with 0.01 ml of *Staphylococcus aureus*. These samples, seeded with *Staphylococcus aureus*, were streaked onto blood agar plates 5 minutes and 7 hours after seeding.

The third set of samples was collected from the bottom of the simulator only, in sterile bottles containing no sodium thiosulfate. TSA pour-plates were made from 1-ml aliquots of the samples. After incubation for 96 hours the number of colonies were counted. Typical fungus colonies were transferred to Sabouraud-dextrose agar slants and plates for identification.

3. Carpet. The carpet was sampled by two methods: (1) using Rodac plates, and (2) macerating 1-cm diameter plugs. The Rodac plates

were of three kinds of culture media; TSA, EMB, and SAB. A total of 84 sites was sampled, 42 from the top of the carpet around the elevator and gear areas, and 42 from bottom of the carpet around the elevator and gear areas. They were then incubated for a 48-hour period, the colonies were counted and the organisms identified.

For use in the maceration method, as shown in Figure 1, four plugs 1 cm in diameter were taken from the elevator and gear area. These were placed in 90 ml of 0.25-percent peptone water and macerated for 2 minutes in a blender. Thirty ml of the extract was added to 90 ml of 0.25-percent peptone water, which resulted in a 1:4 dilution. One ml of the 1:4 dilution was then added to 9 ml of peptone water resulting in a 1:40 dilution. One ml and 0.1 ml of each dilution were plated in duplicate on TSA pour-plates. One one-hundredth ml of each dilution was also streaked (in duplicate) onto blood and EMB plates as shown in Figure 2.

C. Results

The results that were obtained from the air samples indicated the presence of fungi and bacteria. The colony counts for the fungi averaged 12, while the average bacterial count was 67. The fungi present were: _Penicillium_ species, _Aspergillus_ species, and _Fusarium_ species. The bacteria present were _Bacillus_ species and _Micrococcus_ species.

Results obtained from the water samples with sodium thiosulfate are listed in Table 4. Water samples from the same sites were then put into Lactose broth tubes. The results are given in Table 5. Because of the scant growth obtained from the water samples, additional samples were taken. These results are shown in Table 6. Since scant or no growth was again obtained, the water samples were innoculated with _Staphylococcus aureus_ to determine if the chlorine and/or sodium thiosulfate were exhibiting a strong bacteriocidal effect. The samples were streaked onto blood agar plates and incubated. The results obtained are listed in Table 7.

Results of the colony counts on the Rodac plates are listed in Table 8. The colony counts for the carpet maceration are in Table 9.

Samples of granular material from the bottom of the simulator were cultured and the average colony count was 174 colonies/ml. The colonies were found to be fungi and were transferred to Sabouraud slants and plates. After 144 hours incubation the fungus was examined microscopically using a lacto-phenol cotton blue preparation. The organism appeared to be _Allescheria boydii_.

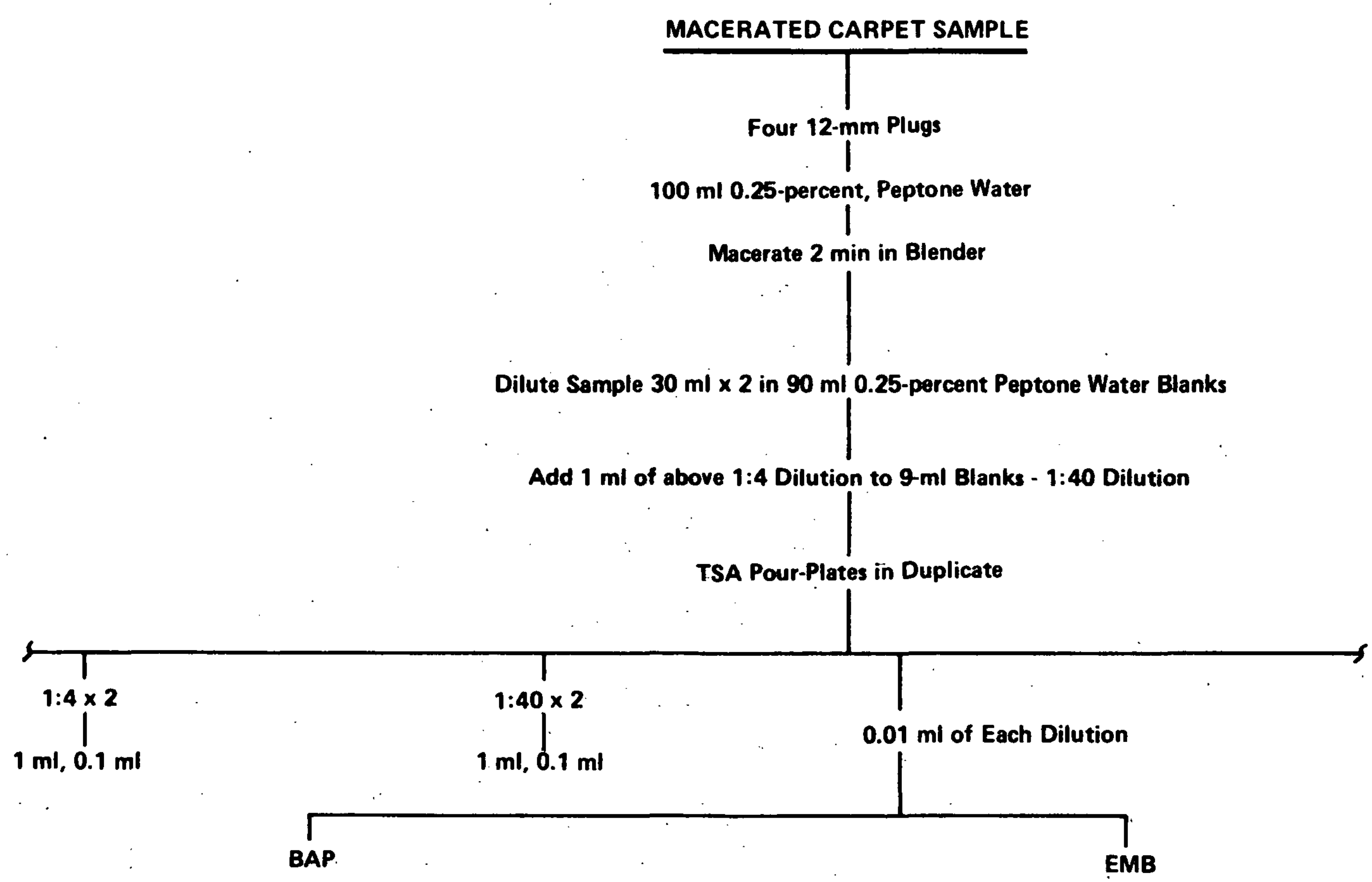

Figure 2. Procedure for maceration of carpet samples.

TABLE 4. WATER SAMPLES COLLECTED WITH 0.02 SODIUM THIOSULFATE PER 100-ml WATER

Sites Sampled	Incubation Time		
	48 Hours	72 Hours	96 Hours
1. Surface	No growth	No growth	No growth
2. Overflow basin	No growth	No growth	No growth
3. Bottom	No growth	76 colonies	1 colony <u>Allescheria boydii</u>
4. Potable water line	No growth	No growth	No growth
5. Television camera	No growth	No growth	No growth
6. Metal slug (from bottom)	No growth	No growth	No growth

TABLE 5. RESULTS OF WATER SAMPLES FROM THE FIRST TEST SET INOCULATED INTO DOUBLE STRENGTH LACTOSE BROTH AND INCUBATED FOR 48 HOURS AT 35° C

Sites Sampled	Results
1. Surface	No acid or gas
2. Overflow basin	No acid or gas
3. Bottom	No acid or gas
4. Potable water line	No acid or gas
5. Television camera	No acid or gas
6. Metal slug (from bottom)	No acid or gas

TABLE 6. SECOND TEST SET OF WATER SAMPLES

Sites Sampled	Incubation Time	
	24 Hours	48 Hours
1. Surface with sodium thiosulfate	No growth	No growth
2. Surface without sodium thiosulfate	No growth	No growth
3. Bottom with sodium thiosulfate	No growth	No growth
4. Bottom without sodium thiosulfate	No growth	No growth

TABLE 7. SAMPLES INOCULATED WITH
0.01-ml STAPHYLOCOCCUS AUREUS

Sites Sampled	Streaked on Blood Agar Plates	
	5 Minutes after Inoculation	7 Hours after Inoculation
1. Surface with sodium thiosulfate	Staphylococcus aureus	Staphylococcus aureus
2. Surface without sodium thiosulfate	Staphylococcus aureus	Staphylococcus aureus
3. Bottom with sodium thiosulfate	Staphylococcus aureus	Staphylococcus aureus
4. Bottom without sodium thiosulfate	Staphylococcus aureus	Staphylococcus aureus

[2]. A fungus resembling Allescheria boydii grossly and microscopically was also isolated from the macerated carpet sample taken from the gear area as shown in Figure 3. Table 10 lists all organisms isolated from the NBS area.

TABLE 8. COLONY COUNT ON CARPET RODAC PLATES

TSA		SAB		EMB	
Elevator Area	Gear Area	Elevator Area	Gear Area	Elevator Area	Gear Area
(Top)					
1. 19	9	70	2	0	0
2. 14	49	16	13	0	0
3. 24	4	15	8	0	25
4. 32	12	9	5	0	0
5. 23	12	6	13	5	0
6. 28	29	15	overgrown	0	0
7. 61	17	14	6	0	0
(Bottom)					
8. 0	0	0	0	0	0
9. 2	0	0	0	0	0
10. 2	0	3	0	0	0
11. 0	1	0	0	0	0
12. 0	1	2	0	0	0
13. 1	0	0	0	0	0
14. 1	0	22	1	0	0

TABLE 9. COLONY COUNTS OF MACERATED CARPET SAMPLES PER cm^2

Sample Area	Colony Count
Elevator entrance	52×10^3
Elevator entrance	67×10^3
Gear area	21.2×10^3
Gear area	18.5×10^3

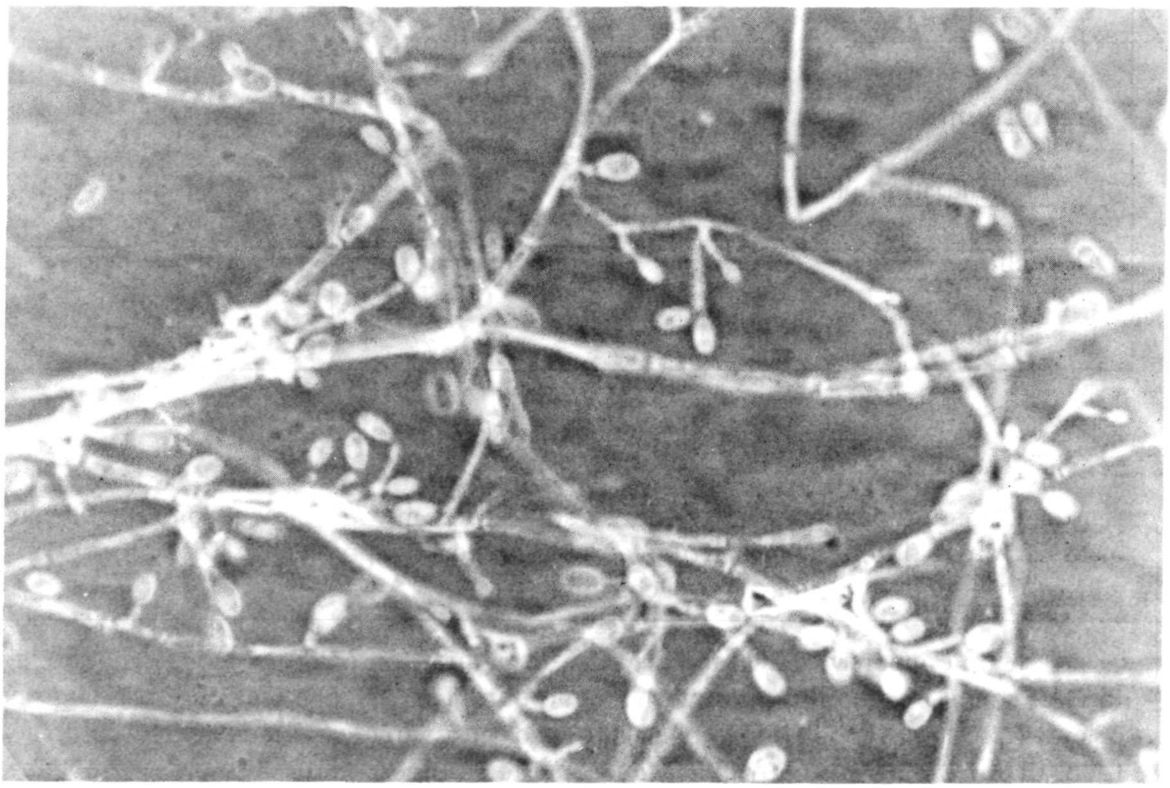

Figure 3. Allescheria boydii.

TABLE 10. ORGANISMS ISOLATED FROM THE NBS AREA

Carpet	Water	Air
Bacillus species	*Allescheria boydii*	*Bacillus* species
Gram pos. non-spore-forming rods	Gram pos. non-spore-forming rods	*Micrococcus* species
Micrococcus species		*Penicillium* species
Staphylococcus epidermidis		*Aspergillus* species
Pseudomonas aeruginosa		*Fusarium* species
Yeast		
Penicillium species		
Hormodendrum species		
Allescheria boydii		
Three unidentified fungi		

D. Discussion

Those organisms which were isolated from the air are considered part of the normal flora of the atmosphere. The counts-per-cubic-foot of air was within an acceptable range, two colonies/ft^3.

The carpet was evaluated using an adaptation of the method of Anderson in Reference 6 in conjunction with Rodac sampling, which was the usual method of culturing the carpet in the past [3]. No data were available to indicate the level of microorganisms which was considered acceptable on carpeted areas such as the NBS deck. The results described were meant to serve a two-fold purpose. One, a comparison was made between the usual Rodac method of sampling and the maceration technique. The results, as shown in Tables 8 and 9, indicated that no correlation can be made between the two methods when attempting to enumerate microorganisms. The same group of organisms were recovered by each method with one exception: a colony of Allescheria boydii was recovered from the macerated sample left at room temperature for 168 hours.

Pseudomonas aeruginosa was found to be associated with the bottom-glued portion of the carpet in both areas.

Water samples appeared free of lactose-fermenting gram-negative rods and the counts were within acceptable limits. The highest counts in the water were found at the bottom of the simulator and Allescheria boydii appeared to be the most numerous organism, 176 colonies/ml in this area.

E. Conclusions and Recommendations

The microbiological quality of the water in the NBS met the standards for swimming-pool water [4]. However, because of the nature of the physical work being carried on in the simulator and the constant possibility of small cuts caused by working under water, the fungus Allescheria boydii was a potential hazard. This organism was found in small numbers on the carpeted deck and was reported to proliferate in various decomposed organic materials. Some sections of the carpet appeared degraded, possibly by constant contact with chlorine from the simulator. This formed an excellent reservoir for the organism which can be washed down into the simulator. Pseudomonas aeruginosa found in the carpet, but not in the water, appeared to be associated with adhesive used to fasten the carpet to the deck. This organism is the causative agent of eye and ear infections associated with swimming [5].

However, since no problems have been associated with these organisms, it appears that the present method of simulator maintenance and sanitation are adequate for safe operation of the simulator.

Replacement of the present carpet with an acceptable covering bound by a bacteriostatic adhesive is desirable.

It is recommended that the monthly surveillance be continued as part of the standard operating procedure for maintenance of the NBS.

SECTION III. CEILCOAT 300 RESISTANCE TO MICROBIAL ATTACK

The inside surface of the NBS was to be coated with Ceilcote 300, a glass flake-impregnated polyester. Ceilcote had been used by various manufacturers for coating tanks containing acids, caustics and chlorine; however, the vendor could supply no data on the effects of microorganisms on Ceilcote. A test protocol using the two organisms isolated from the NBS in the past was established. These organisms, Pseudomonas aeruginosa and Allescheria boydii, were grown in mass cultures and applied under various conditions to samples of coated metal. No changes in the coating were detected at the end of the 6-week test period.

A. Materials and Methods

The Ceilcote Company provided panels of metal 3 × 6 inches coated on both sides with Ceilcote 300 polyester coating.

A series of three beakers was sterilized with a gauze pad placed in the bottom of each. Beakers were covered with aluminum foil; panel samples and support rods were sterilized with ethylene oxide gas.

Cultures of Pseudomonas aeruginosa and Allescheria boydii were grown on trypticase soy agar and malt extract plates, respectively, and the cells were harvested and washed three times in sterile distilled water. The cell concentration was adjusted to 1000 cells/ml. This gave a cell concentration approximately five times higher than the highest level found in the NBS.

A control panel was suspended in the first beaker, the gauze pad was saturated with sterile distilled water and the aluminum foil cover was replaced over the beaker. Each of two other panels was placed in the cell suspensions, allowed to remain for 24 hours, then suspended in the same manner as the control panel. All beakers were incubated for 4 weeks at 30° C and 2 weeks at 25° C. Sterile water was added to the beakers as necessary to keep the humidity in the culture vessels high.

At the end of the 6-week test period all beakers were sterilized by ethylene oxide and the plates examined macroscopically and microscopically for evidence of degradation.

B. Results

Control and treated panels were examined macroscopically and microscopically for signs of discoloration, blistering, and pitting. No difference was noted between the coating treated with bacteria and fungi and the untreated control panels.

The treated panels were autoclaved at 121° C for 20 minutes to determine the effects of high temperatures on the coating. Areas of cracking and blistering were noted. The entire coated surface came away from the metal panels. Base coat and top coat did not part.

SECTION IV. INVESTIGATIONS AFTER RESURFACING TANK

The NBS was drained in February, 1971, in preparation for sandblasting and resurfacing with Ceilcote 300.

The simulator was refilled in May, 1971, and subsequent water samples were free of Pseudomonas aeruginosa and Allescheria boydii and met the requirements for safe swimming-pool water. The carpet on the upper deck gave positive results for Pseudomonas aeruginosa and Allescheria boydii. Water samples were taken from the decompression chamber deluge tank, the interior of which showed signs of advanced corrosion of the zinc lining. This prompted an investigation which showed the water was free of microbiological problems and the corrosion was caused by galvanic action.

A. Materials and Methods

Samples of debris from the simulator bottom were taken by entering the simulator after it was drained. A sterile spatula was used to scrape up the granular debris, which was placed in a sterile beaker covered with foil. A sterile syringe was used to collect 100 ml water from the bottom. The water was placed in a sterile screw-cap medicine bottle.

After the tank had been cleaned, coated, refilled, and the chlorine content adjusted to 1.5 ppm, water samples were collected from the locations shown in Table 11 and cultured in the media indicated.

TABLE 11. WATER SAMPLES COLLECTED FROM NEUTRAL BUOYANCY SIMULATOR FEBRUARY THROUGH JUNE, 1971

Date	Collection Site	Sample Volume	Media
May 18, 1971	Surface water Surface water Surface water	100 ml 100 ml 100 ml	Malt extract agar (MEA) Phenol red lactose broth (PRLB) fermentation tubes Trypticase soy agar plates (TSA) Eosin methylene blue plates (EMB)
May 25, 1971	Surface water Surface water Surface water	100 ml 100 ml 100 ml	Same
May 25, 1971	Bottom water and Bottom sediment	100 ml	Same
June 10, 1971	500 gallon water deluge tank and corrosion residue	100 ml	Same
June 11, 1971	Surface water Surface water Surface water	100 ml 100 ml 100 ml	Same
February 2, 1971	Bottom water Bottom sediment	100 ml	Same

Rodac plates containing trypticase soy agar (TSA) were used on June 11, 1971, to sample the carpet on the upper deck of the NBS in 16 locations, as called out in Table 12.

TABLE 12. RODAC CULTURES ON CARPET OF NEUTRAL BUOYANCY SIMULATOR UPPER DECK, JUNE 1971

Plate	Count	Microbial Content
1	25	Normal flora*
2	13	Allescheria boydii
3	78	Normal flora
4	66	Pseudomonas aeruginosa
5	45	Allescheria boydii
6	33	Normal flora
7	100	Pseudomonas aeruginosa
8	>200	Normal flora
9	>200	Normal flora
10	71	Pseudomonas aeruginosa
11	>200	Normal flora
12	>200	Normal flora
13	>200	Normal flora
14	>200	Allescheria boydii
15	>200	Normal flora
16	>200	Normal flora

* Normal flora — Microorganisms ordinarily found as part of the environment which do not under ordinary circumstances impose a disease condition to man.

B. Results and Discussions

Samples of water and bottom sediment taken in February 1971 contained an average of 250 000 colonies of bacteria and fungi/ml. No coliform bacteria were present, as was indicated by the absence of acid or gas formation in the fermentation tubes. However, Pseudomonas aeruginosa and Allescheria boydii were isolated from the sample.

All water and sediment samples collected in May and June 1971 contained less than 30 colonies of microorganisms/ml. No coliform bacteria, Allescheria boydii, or Pseudomonas aeruginosa were present.

Cultures from the water-deluge tank contained less than 30 colonies of microorganisms/ml. No coliform bacteria, Allescheria boydii or Pseudomonas aeruginosa were present. The pH of the sediment and water in the simulator was six.

Results of Rodac plate cultures taken on July 11, 1971, are described in Table 12. Pseudomonas aeruginosa and Allescheria boydii were isolated from several areas of the carpet.

It was noted that Pseudomonas aeruginosa and Allescheria boydii had been isolated from the carpet and water in the past. The absence of these microorganisms from the water indicated that it had not yet become contaminated from the carpet, an apparent reservoir for the organisms. The NBS water met the requirements for safe swimming-pool water as set forth by the Department of Health, Education and Welfare, Public Health Service Communicable Disease Center-Environmental Health Training Section, Publication Number 665.

It was concluded from studies of the NBS water and carpet that the carpet serves as a reservoir for potential pathogenic microorganisms as Pseudomonas aeruginosa and Allescheria boydii. Therefore, it was necessary to prevent contamination of the freshly-filled and resurfaced simulator by either providing a practical method of sterilizing the existing carpet or removing the carpet and replacing it with a material less susceptible to microbial invasion.

Several alternatives were considered as solutions to the problem.

Ultraviolet treatment of the NBS water and carpet showed ultraviolet light had relatively low-penetration capacities, was detrimental to human health, and had varying biocidal effect on microorganisms, some highly resistant to ultraviolet. Also, proper installation and maintenance of a ultraviolet sterilant system involved appreciable cost.

Replacement of the carpet with synthetic removable rubber or plastic mats was considered. The molecular structure of the materials rendered them susceptible to degradation by chlorine or phenolic disinfectant. Degraded material forms a suitable substrate for fungal growth. Therefore, it would be necessary to periodically replace these mats.

Investigation on the carpet adhesive showed it to support growth of Pseudomonas aeruginosa. This organism is the causative agent of eye and ear infections associated with swimming. From these findings consideration was given to another method of securing carpet to the deck surface. Metal strips around the edge of the carpet to hold it in place were considered.

Microbial studies were carried out on three samples of carpet in order to aid in determining which was best suited for covering the upper deck of the NBS. Astroturf L-10 showed the least amount of microbial accumulation.

Microbes were not observed in the fiber or carpet backing at the end of the 61-day test period. The reduction of microbial population on Astroturf was due in part to resins included in the carpet, which contain formaldehyde.

From these results it was recommended that the present indoor-outdoor carpet on the deck of the NBS be replaced with Astroturf L-10. Astroturf L-10 material was loosely woven, making it less susceptible to microbial buildup, and it could be vacuumed.

It was also recommended that further studies be made as to the effect of prolonged exposure of Astroturf L-10 to chlorine and microorganisms, and that tests be carried out to investigate the feasibility of developing a suitable biocidal spray for use in periodic decontaminations of the Astroturf L-10.

SECTION V. MONITORING PROCEDURE

A monitoring procedure for the NBS was set up in June 1971, based on microbiological problems found peculiar to the simulator. The ensuing work outlines and discusses the procedure.

A. Materials and Methods

Prepare water-sample bottles by adding 0.5 ml of sodium thiosulfate solution (0.15 grams of sodium thiosulfate dissolved in 100 ml of distilled water) to four clean, 4-ounce medicine bottles. Cap the bottles loosely and sterilize at 121° C for 30 minutes.

Collect samples of NBS water when the pool is in use. Take at least two 100-ml samples from the surface of the water and two samples from the bottom by a diver. Collect bottom samples in such a manner to include granular debris from the bottom of the simulator.

Remove the cap from the bottle carefully so that the inner neck of the bottle is not touched while carrying out all water-sampling procedures. Plunge the open bottle under the water and allow it to fill, then replace the cap. Take samples from the bottom by having the diver remove the cap from the bottle at the bottom of the simulator.

When collecting samples, do not rinse the bottles because the sodium thiosulfate will be lost.

Take the sample bottles immediately to the laboratory and culture within 20 minutes after collection. If not cultured immediately the samples are stored at 5° C.

Examination of pool water is conducted in the following manner:

1. Detection of Coliform Organisms.

   a. Presumptive test for coliforms. Inoculate into each of 5 double strength 10-ml phenol red lactose broth fermentation tubes, 10 water samples which have been shaken 25 times. Incubate 24 hours at 35° C.

   b. Confirmed test. Subculture positive lactose broth tubes (acid and gas production) onto three EMB plates 0.01-ml streak and incubate for 24 hours at 35° C. Incubate negative or doubtful tubes an additional 24 hours.

   c. Completed test. Pick and transfer colonies typical of coliform bacteria to differential medium for identification of gram-negative rods. A gram stain is made to confirm the presence of gram-negative rods.

2. Standard Plate Count. Plate 1-ml water samples in triplicate from each bottle of water. Pour 10- to 15-ml TSA, cooled to 45° C, onto each water aliquot, mixed and allowed to solidify. Incubate the plates for 24 to 48 hours at 36° C and count the colonies.

3. Carpet Cultures. These are made by use of Rodac plates filled with the following media: Trypticase Soy Agar (TSA), Levine Eosin Methylene Blue Agar (EMB), and Sabouraud Dextrose (SDA). Take cultures from 20 random sites on the top and bottom of the carpet using a total of 60 plates (20 filled with each type of medium).

Incubate TSA and EMB plates for 48 hours at 35° C and SDA plates for 14 days at room temperature, then examine plates for bacteria and fungi of sanitary importance and count colonies.

B. Interpretation and Reporting of Results

1. Coliform Test. Not more than 15 percent of the samples over a 3-month period should show positive results in any of the five 10-ml test fermentation tubes.

2. Standard Plate Count. Not more than 15 percent of the plates over a 3-month period should show more than 200 bacterial colonies/ml.

3. Rodac Plates. No standard has been set for interpretation of results from carpet samples monitored by use of Rodac plates. Examine plates for total charted microbial counts and then establish a normal for the NBS upper deck. These counts taken over a 1-year period will show the trend for microbial loads on the deck. Organisms which are important from a sanitation viewpoint shall be isolated and identified.

4. Results. Report the results of monthly NBS cultures by means of interdepartmental correspondence and issue a technical report quarterly giving details of the work.

C. Discussion and Recommendations

Diver safety from the microbiological viewpoint is the primary reason for monitoring the NBS. The second reason is to detect the presence of microorganisms which may degrade the lining and carpeted area of the simulator.

Table 13 is a list of microorganisms which are considered undesirable to have in the simulator or the simulator environment. Some of the microorganisms listed in Table 13 were found in the simulator or on the deck. The June survey showed that the simulator water was free of undesirable microorganisms. The carpet, however, harbored Pseudomonas aeruginosa and Allescheria boydii. Removing the carpet and scrubbing away the old adhesive will greatly aid in keeping the water from being continuously contaminated.

Table 14 is a list of all microorganisms isolated from the simulator.

TABLE 13. MICROORGANISMS WHICH ARE OF SANITARY IMPORTANCE

Organism	Problem
Allescheria boydii	Utilize rubber or wood in life cycle. Can cause infection in man.
Fusarium species	Utilize rubber or wood in life cycle. Can cause infection in man.
Hormodendrum species	Utilize rubber, plastics or wood in life cycle.
Pseudomonas species	Attacks rubber. Can cause infection in man.
Escherichia coli	Presence indicates human fecal pollution of water.
Staphylococcus aureus	Causes infection in man.
Serratia species	Causes infection in man.

TABLE 14. MICROORGANISMS ISOLATED FROM THE NEUTRAL BUOYANCY SIMULATOR IN A 2.5-YEAR PERIOD

Microorganism	Location
Allescheria boydii	Water, carpet, rubber mats
Fusarium species	Carpet, sponge rubber in tank
Alternaria species	Carpet
Aspergillus species	Carpet
Penicillium species	Carpet
Rhizopus species	Carpet, styrofoam
Pseudomonas species	Carpet, water, sponge rubber in tank
Streptomyces species	Air
Bacillus species	Carpet, water
Micrococcus species	Carpet, water
Rhodotorula species	Carpet
Hormodendrum species	Carpet

SECTION VI. NUTRIENT CHARACTERISTICS OF SODIUM CITRATE IN NEUTRAL BUOYANCY SIMULATOR WATER

Tests were conducted to determine the nutrient characteristics of 150 ppm of sodium citrate additions to NBS water which contained 1.5 ppm. Three species of bacteria and four species of fungi were tested. Two of the fungi were species found in the NBS area as environmental contaminants, but not readily identifiable; therefore they were labeled as unidentified species numbers one and two.

A. Materials and Methods

Samples of NBS water were received in two 4-liter containers. The first container labeled "Sample A" contained 1.5-ppm chlorine at a pH of 7.8. The second container labeled "Sample B" contained 1.5-ppm chlorine and 150 ppm sodium citrate at a pH of 7.2.

A set of six Erlenmeyer flasks (500 ml each) was sterilized and 250 ml of Sample A were added to each of three flasks. The other three flasks were each used to contain 250 ml of Sample B. Table 15 shows the inoculation procedure used for each set of flasks.

Cultures of Escherichia coli, Staphylococcus aureus and Proteus mirabilis were grown for 18 hours in trypticase soy broth at 35° C. The cultures were centrifuged and washed twice with sterile distilled water before diluting and adjusting the cell concentration to approximately 10 000 organisms/

TABLE 15. INOCULATION PROCEDURE FOR NBS WATER AND CITRATED NBS WATER

Flask	Water Content	Inoculum
A-1	1.5-ppm chlorine	None — Control
A-2	1.5-ppm chlorine	Penicillium, Aspergillus Unidentified species 1 & 2
A-3	1.5-ppm chlorine	Eschericia coli Staphylococcus aureus Proteus mirabilis
B-1	1.5-ppm chlorine plus 150-ppm sodium citrate	None — Control
B-2	1.5-ppm chlorine plus 150-ppm sodium citrate	Penicillium, Aspergillus Unidentified species 1 & 2
B-3	1.5-ppm chlorine plus 150-ppm sodium citrate	Escherichia coli Staphylococcus aureus Proteus mirabilis

ml. Two and five-tenths milliliters of this suspension was used for inoculum to give a final concentration of approximately 100 organisms/ml in the test flasks.

Fungus spores were harvested by scraping spores from petri plate cultures mixed in sterile distilled water and by dispensing 2.5 ml in the test flasks.

The flasks were capped and incubated at 31° C (NBS water temperature) for 8 days. At the end of this time all flasks were examined macroscopically for turbidity. Subcultures were made to blood agar plates and eosin methylene blue agar from each flask listed in Table 15.

Turbidity was observed in flask B-3 in the first test. Therefore, this test was repeated using a freshly-prepared water sample to verify growth.

B. Results and Discussion

After 8 days incubation at 31° C no bacterial growth was detected by subculture to the recovery media from test flasks in Group A which contained only 1.5 ppm of chlorine. In Group B, no growth was observed in recovery media from flasks B-1 or B-2. However *Escherichia coli* and *Proteus mirabilis* were recovered from flask B-3. The repeat test of flask B-3 using a second water sample plus sodium citrate gave the same results as the first test, growth of *Escherichia coli* and *Proteus mirabilis*.

The results of this procedure indicate that NBS water containing 1.5 ppm chlorine is inhibitory to the microorganisms listed in Table 15. It appears, however, that the addition of sodium citrate and buffering agents to chlorinated NBS water supports growth of the bacteria used in this study. The growth of fungi is not reported because of the short time span alloted to this initial study.

Bacterial growth is significant because no growth was observed in the water containing chlorine only. Growth in the chlorine-sodium citrate solution indicates that the sodium citrate was the growth-promoting factor since its addition was the only change in the test solution.

SECTION VII. MICROBIOLOGICAL TESTS MADE JULY THROUGH OCTOBER, 1971

The NBS water and upper simulator deck were checked to see if they were safe for personnel working in the area. Water samples were taken from four sites in the NBS each month from July through October, 1971. Rodac-plate cultures were made from 30 random sites on the upper deck in September. Results of these microbiological tests showed the water and area was safe for personnel.

A. Materials and Methods

Water sample bottles were prepared by adding 0.5 ml of sodium thiosulfate solution (1.5 grams of sodium thiosulfate dissolved in 100 ml distilled water) to four clean 4-ounce (28.4-gram) medicine bottles. The bottles were loosely capped and sterilized at 121° C for 30 minutes.

Samples of NBS water were collected from the surface of the water and two samples were taken from the bottom by a diver. Bottom samples were collected in such a manner to include any granular debris from the bottom of the simulator.

In carrying out all water-sampling procedures, the cap from the bottle was carefully removed so that the inner neck of the bottle was not touched. The open bottle was then plunged under the water and allowed to fill and the cap replaced. Samples from the bottom were taken by having the diver remove the cap from the bottle at the bottom of the simulator.

The sample bottles were taken immediately to the laboratory and cultured.

Examination of pool water was conducted according to the Section V monitoring procedure.

B. Results

Average microbial counts from surface samples over the period of July through October 1971, was 1 colony/ml. Counts from the bottom of the simulator averaged 4 colonies/ml.

Table 16 gives the monthly counts and coliform report for each month during the July through October reporting period. Table 17 shows bacterial and fungus counts from 30 random sites on the metal NBS deck.

TABLE 16. JULY THROUGH OCTOBER MICROBIAL COUNTS IN THE NEUTRAL BUOYANCY SIMULATOR

Month	Surface Sample Count		Bottom Sample Count		Coliforms	
	No. 1	No. 2	No. 1	No. 2	Surface	Bottom
July	zero	zero	4	4	zero	zero
August	zero	zero	4	5	zero	zero
Sept.	zero	2	3	2	zero	zero
Oct.	2	2	5	7	zero	zero

TABLE 17. RODAC PLATE RESULTS FROM THE NEUTRAL BUOYANCY SIMULATOR METAL DECK

Plate No.	Trypticase Soy Agar Count	Sabouraud Agar Count	Eosin Methylene Blue Agar Count
1	25	14	zero gram negative
2	37	7	zero gram negative
3	15	9	zero gram negative
4	56	11	zero gram negative
5	102	5	zero gram negative
6	83	1	zero gram negative
7	61	6	zero gram negative
8	80	16	zero gram negative
9	83	23	zero gram negative
10	74	3	zero gram negative

Bacteria and fungi found on trypticase soy agar and Sabouraud agar were of the type normally found on environmental surfaces. None of those microorganisms isolated during the July through October reporting period were of sanitary importance. No gram-negative bacteria were found on the eosin methylene blue plates. This indicated the absence of any great amount of soil pollution or unsanitary conditions on the metal deck.

SECTION VIII. MICROBIOLOGICAL GROWTH STUDIES OF THE NEUTRAL BUOYANCY WATER CONTAINING RUST INHIBITOR ADDITIVES

A series of microbial growth support tests was performed in order to determine the possible nutrient properties of corrosion-inhibiting chemicals in NBS water. Addition of sodium phosphate (NaH_2PO_4), sodium silicate ($NaSiO_4$), and sodium nitrate ($NaNO_3$) in varying concentrations to NBS water and distilled water (control system) was made by the chemistry laboratory. Bacterial and fungus cultures were added to portions of the water and incubation was carried out at 30° C. Results showed that although no gross bacterial growth took place, the chemicals acted as a holding solution and some of the organisms survived without multiplying and could be recovered when subcultured to nutrient media.

A. Materials and Methods

Water samples were received from the NBS in five 4-liter bottles. Each was labeled with the chemical content. Control samples of distilled water had the same chemical content as the NBS water. The chlorine content of the NBS water samples was 1.5 ppm. Samples of distilled water contained no chlorine. Table 18 gives the chemical additives and pH values of the NBS and distilled water samples.

Chlorine content of each NBS sample was 1.5 ppm. The pH values were determined by use of pH paper. A 100-ml aliquot of water from each bottle was transferred to each of 20 sterile medicine bottles. These were placed in a 30° C incubator until inoculation with the test organisms.

Test organisms were prepared in the following manner:

TABLE 18. CHEMICAL ADDITIVES AND pH VALUES IN WATER SAMPLES

Specimen Number	pH	Chemical Content
1 Test	7	NBS water; no chemicals
2 Test	7	NBS water/ NaH_2PO_4 25 ppm, $NaSiO_4$ 50 ppm
3 Test	7	NBS water; NaH_2PO_4 25 ppm, $NaSiO_4$ 50 ppm, $NaNO_3$ 10 ppm
4 Test	7	NBS water; NaH_2PO_4 25 ppm, $NaSiO_4$ 50 ppm, $NaNO_3$ 25 ppm
5 Test	7	NBS water; NaH_2PO_4 25 ppm, $NaSiO_4$ 50 ppm, $NaNO_3$ 50 ppm
6 Control	7	Distilled water; no chemicals
7 Control	7	Distilled water; NaH_2PO_4 25 ppm, $NaSiO_4$ 50 ppm
8 Control	7	Distilled water; NaH_2PO_4 25 ppm, $NaSiO_4$ 50 ppm, $NaNO_3$ 10 ppm
9 Control	7	Distilled water; NaH_2PO_4 25 ppm, $NaSiO_4$ 50 ppm, $NaNO_3$ 25 ppm
10 Control	7	Distilled water; NaH_2PO_4 25 ppm, $NaSiO_4$ 50 ppm, $NaNO_3$ 25 ppm

Bacterial cultures of Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa and Staphylococcus aureus were grown for 24 hours in trypticase soy broth (TSB) to a concentration of approximately 10^7 organisms/ml. Each culture was washed three times with sterile distilled water to remove the TSB. A final aqueous suspension of approximately 10^3 cells was made.

Cultures of Penicillium species, Fusarium species, and Aspergillus species were grown on Sabouraud dextrose agar slants. After 7 days incubation, 10-ml sterile distilled water was added to each slant and the culture bottle was shaken to dislodge the fungus spores. These suspensions were pooled and adjusted to a final concentration of approximately 10^3 spores/ml. Each of 10 of the samples placed in medicine bottles was inoculated with 1 ml of the mixed 10^3 cells/ml of bacterial suspension. The other 10 sample bottles were inoculated with 1 ml of the mixed 10^3 cells/ml of fungus spore suspension. All 20 culture bottles were incubated for 4 weeks at 30° C.

Samples were examined daily for visible signs of growth. At the end of 4 weeks the samples were removed from the incubator and portions were transferred to trypticase soy agar, eosin methylene blue and Sabouraud dextrose agar plates. After incubation the plates were examined.

B. Results and Discussion

The addition of the chemicals listed in Table 18 to NBS and distilled water apparently provided a buffer solution in which the test microorganisms could survive. No visible turbidity, indicating extensive growth, was seen. However, after 4 weeks incubation at 30° C, subculture showed that all the organisms survived with the exception of Staphylococcus aureus. From these results it appeared that although the solutions did not support bacterial growth they do allow the microorganisms to remain in a viable condition. In this state the microbes are capable of proliferating when they come in contact with a suitable growth substrate such as rubber, plastic, or other organic material.

SECTION IX. INVESTIGATION OF ALLESCHERIA BOYDII AFTER REFILLING SIMULATOR

Monthly cultures taken from the NBS, after it was drained, sandblasted, resurfaced with Ceilcoat 300 and refilled in May 1971, showed the total microbial count was less than 10 microorganisms/ml. In November 1971, the water was sampled at more frequent intervals because of the increased swimmer load.

On January 7, 1972, it was noted that the bottom samples contained a larger amount of granular debris. Microscopic examination of the material showed many fragments of diatoms in the sediment. Cultures showed

Allescheria boydii was present in numbers greater than 200 colonies/ml. Previous studies had shown that Allescheria boydii was associated with rubber, plastic and other organic materials found in the simulator. The presence of the fungus this time appeared to be associated with diatomaceous earth debris on the bottom of the simulator.

A. Materials and Methods

Water samples were collected from the NBS according to the procedure outlined in Section V.

Examination of water samples was conducted in the following manner:

1. Detection of Coliform Organisms.

a. Presumptive test for coliforms. Inoculate into each five double strength 10-ml phenol red lactose broth fermentation tubes, 10-ml water samples which have been shaken 25 times. Incubate 24 hours at 35° C.

b. Confirmed test. The negative tubes were incubated an additional 24 hours.

c. Completed test. A gram stain was made.

2. Standard Plate Count. One-ml water samples were plated in triplicate from each bottle of water. Ten- to fifteen-ml TSA cooled to 45° C were poured onto each water aliquot, mixed, and allowed to solidify. The plates were incubated for 48 hours at 35° C and the colonies counted.

3. Upper-deck Cultures. These are made by use of Rodac plates filled with trypticase soy agar (TSA) and Levine eosin methylene blue agar (EMB). Cultures were taken from 20 random sites on the metal deck and Astroturf.

TSA and EMB plates were incubated for 48 hours at 35° C. The plates were examined for bacteria and fungi of sanitary importance and the colonies counted.

4. Additional Cultures. These were made from chunks of diatomaceous earth removed from the filters during the backwashing process. These pieces of filter material were ground in a sterile mortar and each gram of

material was diluted in 10-ml sterile distilled water. Cultures were made in the same manner as the Standard Plate Count described in 2 above.

B. Results

All cultures made for the detection of coliform organisms from November 1971 to January 1972 were negative. Negative coliform counts indicate the absence of human fecal pollution in the NBS. All water samples from the surface of the NBS consistently grew less than 10 colonies of bacteria/ml[3]. Water samples taken from the bottom of the pool grew less than 10 colonies of bacteria/ml until the first sample was taken in January. At the time of sampling it was noted that the water in the lower half of the NBS was dark green. The sample obtained by the diver contained a large amount of debris which, when examined microscopically, was found to be composed of a great proportion of diatoms and other silica-like fragments. Cultures made from this specimen yielded colony counts of greater than 200/ml of *Allescheria boydii*, as presented in Table 19. Cultures made during the following weeks showed decreasing numbers of *Allescheria boydii*. This was also accompanied by a sediment in the pool. A sample from the bottom, taken on January 24, 1972, again contained a large amount of sediment composed of many diatomaceous earth-fragments. The count, as shown in Table 20, was greater than 200 colonies/ml of *Allescheria boydii*.

Table 20 gives results of cultures taken from various areas on the first floor of the NBS building. The city water, supplying the building and filters, contained only six bacterial colonies/ml and no *Allescheria boydii*. The cement floor next to the filters, which had diatomaceous earth particles on it, yielded a few cultures of *Allescheria boydii*.

Rodac-plate cultures were taken from Astroturf and the metal upper deck of the NBS before and after cleaning the Astroturf. Results shown in Table 21 indicate that the cleaning procedure was not effective in reducing bacterial numbers. However, counts were high only in front of the elevator where most of the dirt from shoes is shed. Otherwise, the counts are within an acceptable range.

TABLE 19. WATER SAMPLES FROM NEUTRAL BUOYANCY SIMULATOR

Sample Date	Surface Sample		Bottom Sample	
	Phenol Red Lactose Broth	TSA Pour Plates	Phenol Red Lactose Broth	TSA Pour Plates
Nov. 22, 1971	No acid or gas	<10/ml	No acid or gas	<10/ml
Dec. 2, 1971	No acid or gas	<10/ml	No acid or gas	<10/ml
Dec. 9, 1971	No acid or gas	<10/ml	No acid or gas	<10/ml
Dec. 21, 1971	No acid or gas	<10/ml	No acid or gas	<10/ml
Jan. 7, 1972	No acid or gas	<10/ml	No acid or gas	>200/ml *Allescheria boydii* (Large amount of debris)
Jan. 13, 1972	No acid or gas	<10/ml	No acid or gas	18 colonies 6 colonies *Allescheria boydii*
Jan. 17, 1972	No acid or gas	<10/ml	No acid or gas	4 colonies *Allescheria boydii*
Jan. 24, 1972	No acid or gas	zero	No acid or gas	zero
Jan. 27, 1972	No acid or gas	<10/ml	No acid or gas	>200/ml large amount of debris

TABLE 20. SAMPLES FROM FILTER AREA

Source	Count	Organisms
Diatomaceous earth from bag	63/0.5 gm	Normal Environmental Flora (NEF)*
Slurry from booster tank	>200/ml	NEF
Water directly from filter	zero/ml	- -
Water from city water line	6/ml	NEF
Rodac-plates from area adjacent to filters		
1. Rubber floor mat	102	NEF
2. Cement floor clear metallic side of filter	>200	NEF
3. Tank	4	NEF
4. Rubber hose on deck	zero	- -
5. Cement floor covered with diatomaceous earth	50	2 colonies <u>Allescheria</u> <u>boydii</u>
6. Cement floor covered with diatomaceous earth	77	7 colonies <u>Allescheria</u> <u>boydii</u>
7. Cement floor covered with diatomaceous earth	94	NEF
8. Top of metal 5-gallon drum	15	NEF

* Normal Environmental Flora (NEF) are those microorganisms usually found on surfaces, and which cause no disease in man or known material deterioration.

TABLE 21. RODAC PLATE RESULTS FROM NBS UPPER DECK BEFORE AND AFTER CLEANING

Location Desired	Colony Count Per 16 cm^2		Comments
	Before	After	
Astroturf (front of elevator)	128	>200	No coliform organisms or *Allescheria boydii* isolated from the 20 sites sampled.
Astroturf (under control console)	71	69	
Astroturf (poolside)	101	113	
Astroturf (under decompression chamber)	26	16	
Metal frame on decompression chamber	17	21	
Astroturf (under bunk)	4	17	
Astroturf (random)	39	66	
Astroturf (random)	28	39	
Astroturf (random)	78	41	
Metal deck	19	23	
Metal deck	31	37	
Metal deck	15	19	
Astroturf (Scuba-gear area)	17	14	
Astroturf (Scuba-gear area)	9	18	
Astroturf (Scuba-gear area)	29	36	
Astroturf (Scuba-gear area)	14	21	
Astroturf (Scuba-gear area)	11	9	
Rubber flipper	19	38	
Rubber flipper	21	24	
Rubber flipper	43	56	

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APPROVAL

MICROBIOLOGICAL ASSAY OF THE MSFC NEUTRAL BUOYANCY SIMULATOR

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The information in this report has been reviewed for security classification. Review of any information concerning Department of Defense or Atomic Energy Commission programs has been made by the MSFC Security Classification Officer. This report, in its entirety, has been determined to be unclassified.

This document has also been reviewed and approved for technical accuracy.

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