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ACTINOMYCETES OF SEWAGE-TREATMENT PLANTS

Municipal Environmental Research Laboratory
Office of Research and Development
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Cincinnati, Ohio 45268

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ACTINOMYCETES OF SEWAGE-TREATMENT PLANTS

by

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FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, noise, and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment--air, water, and land. The Municipal Environmental Research Laboratory contributes to this multidisciplinary focus through programs engaged in

- studies on the effects of environmental contaminants on the biosphere, and
- a search for ways to prevent contamination and to recycle valuable resources.

As part of these activities, the study described herein presented the isolation of numerous strains of actinomycetes from nuisance foams at activated sludge plants, demonstrated in the laboratory that Nocardia amarae may cause the kind of foam observed in the plants, studied factors affecting the growth of N. amarae, and proposed a method of control of the foam by addition of digester supernatant to the activated sludge tanks.

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ABSTRACT

In some sewage-treatment plants of the activated sludge type, a thick foam may be formed at the surface of the secondary aeration and settling tanks. Such foams have often been found to be rich in actinomycetes. This report covers the work done on this problem between April 1971 and May 1974.

Over 250 strains of actinomycetes have been isolated from foams or activated sludge from 19 different sewage-treatment plants located in 8 states. The actinomycete most commonly associated with foams is a previously undescribed Nocardia which has been given the name N. Amarae. It has been demonstrated experimentally in the laboratory that N. amarae may cause the kind of foam observed in the plants.

Factors affecting the growth of N. amarae have been studied and a method was proposed for control of the foam by addition of digester supernatant to the activated sludge.

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We thank the operators of all the plants listed in Table 1 who have been most cooperative in furnishing samples for microbiological analyses.

Mr. Paul E. Wyszkowski, P.E., has carried out the comparison of the operation data of the wastewater treatment plants of Ocean Township, Middletown Township and Bernardsville. In turn we are indebted to the Sewerage authorities of these municipalities for placing their data at his disposal.

The microbiological studies here reported were carried out by Mrs. Mary P. Lechevalier with some assistance by Dr. C. E. Lee. We are greatly indebted to Dr. Francois Mariat of the Institut Pasteur of Paris for testing the pathogenicity of representative sewage Nocardias.

SECTION I

CONCLUSIONS

Thick foams formed on the surface of secondary aeration and settling tanks in sewage-treatment plants of the activated sludge type are often, but not always, rich in actinomycetes.

The dominant actinomycetes in these foams are usually members of the genus Nocardia. Some human pathogens, such as N. asteroides and N. caviae, may occasionally be involved but more often the dominant organism is N. rhodochrous or even more frequently, a new species which we have described as N. amarae.

Pure cultures of N. amarae were added to non-foaming sludge under laboratory conditions and thick foams were produced which were similar to those found in the plants thus proving the etiologic role of the Nocardia.

The operation data for two non-foaming plants and one with a foaming problem were compared. The only obvious difference noted was that in the non-foaming plants the anaerobic digester supernatant was returned without treatment into the system. In the foaming plant, the supernatant was oxidized with chlorine (Purifax treatment) before being returned to the system.

The supernatant from the anaerobic digesters of two different plants were found to contain a nocardiotoxic principle which completely prevented the growth of N. amarae when diluted 1 to 10^{-3} and which was still partially inhibitory at a dilution of 10^{-6}.

It was thus concluded that the return of the nocardiotoxic anaerobic digester supernatant into the sewage treatment system might be an effective way of preventing the formation of nocardial foams.

SECTION II

RECOMMENDATIONS

The production of actinomycetic foams should be prevented because the growth of nocardias in aeration tanks is a health hazard due to the formation of nocardia-containing aerosols and, in addition, the production of thick foams in activated sludge type plants interferes with good sewage treatment and is a source of extra labor costs.

We feel that we may have discovered a proper method of control and we recommend that two further types of studies be carried out to prove this point: 1) pilot studies in cooperating plants equipped with anaerobic digesters, in which the value of returning anaerobic digester supernatant will be explored; 2) a study of the nature of the nocardiotoxic principle found in the supernatant of anaerobic digesters.

In addition, in plants affected with nocardial foam, the foam should be skimmed off the secondary settling tanks and sent to the anaerobic digester. We understand, however, that many plants are designed in such a way that this operation may not be possible.

SECTION III

INTRODUCTION

GENERAL

Ideally, sewage-treatment plants should run smoothly, raw sewage entering at one end and an effluent, not damaging to the environment, coming out at the other end. In practice, plant operators have to face and master numerous crises which may be brought about by excessive rain or by the dumping of large amounts of specific wastes in the sewage to be treated. The resulting disturbances may take many forms. For example, in the case of activated sludge plants, one troublesome "disease" is "bulking" usually associated with a bloom of Sphaerotilus. When bulking occurs, the sludge does not settle and it is impossible to obtain a clear effluent by simple gravity sedimentation (Wells and Garrett, [19]).

Another "disease" of the activated sludge is the formation of foam on the surface of the aeration tanks. This foam, which is less dense than water, floats on the surface of the settling tanks. It may become very thick, 6 inches to one foot is not uncommon, but it may be even thicker. The foam that we are discussing should not be confused with detergent suds. The foam is quite rigid, and where it accumulates, a bucket may be dropped from a height of several feet onto the surface of the foam without passing through it. The formation of such foams is a nuisance, preventing normal gas exchange at the interface and requiring many hours of labor on the part of cleaning crews. Often, no sooner is an aeration tank cleaned than the cleaning must be repeated. The foam may escape from the treatment tanks and be distributed as smelly pieces by the wind. It has been found by Dr. R. F. Lewis that such foams may be full of branching hyphae of actinomycetes. As a matter of fact, one could say that in some cases the foam is an actinomycetic mycelial mat with entrapped air bubbles.

OBJECTIVE

The purpose of this study was to determine, 1) which actinomycetes were found in these foams, 2) the role they played there, and 3) what could be done to prevent the formation of foams.

SECTION IV

MICROBIOLOGICAL SURVEY OF FOAMS

Direct microscopic examination of sludge and foam.

The presence of actinomycetes in the foam and in the sludge from foaming tanks is very easy to demonstrate. It is enough to place a small sample in a drop of water located between slide and coverslip to be able to visualize the numerous hyphae with a phase contrast microscope. In the field, visualization can be made in bright field by staining lightly with methylene blue or cotton blue. The actinomycetic hyphae bear no sporulating structures but are usually banded and may be very wide (2 μ).

The actinomycetes may be almost the only visible organisms in the foam. In the sludge from foaming tanks, actinomycetes are abundantly mixed with other bacteria and protozoa. Occasionally yeasts can also be seen. The presence of Sphaerotilus spp. can also be easily detected by this method.

Direct microscopic examination is an excellent and rapid diagnostic method for the detection of sewage actinomycetic foam. As a matter of fact, if one can detect numerous actinomycetic hyphae in a sample of activated sludge from a non-foaming aeration tank, one may predict that a foaming episode may soon occur.

Geographic distribution of sewage actinomycetic foam.

Dr. R. F. Lewis drew our attention to a number of plants which were suffering from foaming problems. These included the Metropolitan Jones Island Plant at Milwaukee, a plant in San Jose, California, and plants in Miami, Florida, San Antonio and Austin, Texas, and Ocean Township, New Jersey. Actinomycetes were found in the foam from all these plants. Dr. Lewis also put us in contact with Mr. Paul E. Wyszkowski, an engineer familiar with the Ocean Township plant.

Mr. Wyszkowski made a list of 49 sewage-treatment plants of the activated sludge type located in New Jersey. A letter was sent to all the operators of these plants asking if they were affected by the formation of foam. About a dozen operators responded affirmatively. On the basis of phone conversations, some plants were considered especially promising and were visited. Examination of the foam was made in situ and the microscopic demonstration of the actinomycetes to plant operators was always a success. The following plants were visited and were found to have actinomycetic foam: Bedminster Inn, Bordentown Township, Chatham/Madison, Delran, East Windsor, Evesham, Matawan, Ocean Township and Roxbury. In the plant in Middletown Township there was no foaming but a few actinomycetes could be seen in the sludge particles. Foam without actinomycetes but with Sphaerotilus was found at the Morristown (Florence Avenue) plant and one incident of foaming with a lot of Sphaerotilus and very few actinomycetes was observed at the Ocean Township plant.

Samples of foam from out-of-state found to contain actinomycetes were received from: San Jose, California; Hartford and Enfield, Connecticut; the Andover and Riverdale plants in Miami, Florida; Jamaica Bay, New York; Austin, Texas; Arlington, Virginia; and Milwaukee, Wisconsin. Only Sphaerotilus and no actinomycetes were observed in a sample from the Newton Creek Plant of New York City. New Jersey plants without foaming problems which were visited in 1971 and 1972 included those at Bernardsville, the Somerset-Raritan plant in Somerville and that of the American Cyanamid Co. in Bound Brook. During most of this study, the Bernardsville plant was used as a source of sludge visually and analytically free of actinomycetes.

The following general conclusions can be drawn from this survey:

1) Plants with actinomycetic foams can be found in widely different areas.

2) Although thick foams are not invariably associated with the presence of actinomycetes, these organisms are a very common cause of this troublesome problem.

3) From information received from the plant operators, it seems that actinomycetic foaming occurs mainly when the weather is warm, the aeration rate is high and the mixed liquor thick.

Isolation of actinomycetes from foams

Foams were added to different liquid media, both shaken and stationary which were known to support, in some cases, selectively, the growth of soil actinomycetes (El-Nakeeb and Lechevalier [2]. Except for potato-dextrose (PD) these showed overgrowth of the foam actinomycete by other microbes (fungi, bacteria and protozoa) present. Direct plating of the diluted foam was more successful. The actinomycetes grew very slowly only on certain isolation media (1-3 weeks at 28°. These media included "synthetic" agar, chitin agar (El-Nakeeb and Lechevalier [2]), tap water agar, thin Pablum agar (Lechevalier [15]), nutrient agar, and peptone-yeast agar (PY) (Kolstad and Bradley [11]). The best media for isolation (growth in 5-7 days), were found to be Czapek's agar amended with 0.2% yeast extract (YCZ) (Higgins and Lechevalier [9]) and glycerol agar (Gordon and Smith [8]).

Foams were also purified by differential centrifugation and thoroughly washed to concentrate the actinomycetic hyphae prior to plating and remove some of the associated biota.

Identity of the actinomycetes present in foams

Samples of sludge and/or foam were received or collected from the plants listed in Table 1. For our taxonomic study, strains listed in Table 2 were used. The methods were as described in Lechevalier, M. P. and Lechevalier, H. A. [17].

When the physiology of certain of the sewage strains was compared with that of strains of N. asteroides, N. brasiliensis, and N. caviae, it was found to differ as indicated in Table 3. These sewage organisms were closest to N. ("Mycobacterium") rhodochrous, strains of which are also isolated from some foams. A comparison of these two taxa, along with the reactions of strains of N. rhodochrous isolated from sewage, N. vaccinii 3500, and N. carnea 3419, are presented in Table 4.

The principal characteristics differentiating the new organism from N. rhodochrous are the following: nocardomycolic acid type, hydrolysis of tyrosine, growth at 10 C, growth on adenine and on 5% NaCl, acid from inositol, maltose, rhamnose, salicin, and sorbitol, utilization of benzoate and citrate, and survival at 50 C for 8 h. The principal differences between the new organism and Gordon's (Gordon [4]) rhodochrous-related Nocardia sp. (which she placed in

Table 1. ACTINOMYCETES ISOLATED FROM FOAM AND/OR ACTIVATED SLUDGE

Location		Predominant actinomycetes	Anaerobic digester
California	San Jose	Nocardia rhodochrous	
Connecticut	Hartford	Nocardia rhodochrous	
	Enfield	Nocardia amarae	-
Florida	Miami (Andover and Riverdale plants)	Nocardia amarae, N. asteroides	-
New Jersey	Bedminster Inn	Streptomyces, Micromonospora, Nocardia asteroides, Actinomadura sp.	-
	Bordentown Township	Nocardia amarae, N. rhodochrous	-
	Chatham/Madison	Nocardia amarae	+ [a]
	Delran	Nocardia amarae, N. asteroides, N. rhodochrous	-
	East Windsor	Nocardia caviae, N. asteroides	-
	Evesham	Nocardia amarae	-
	Matawan	Nocardia amarae	-
	Middletown (No foam)	Nocardia rhodochrous	+ [b]
	Ocean Township	Nocardia amarae	+ [c]
	Roxbury	Nocardia rhodochrous	-
New York	Jamaica Bay	Nocardia amarae	+ [a]
Texas	Austin	Nocardia amarae, N. asteroides	-
Virginia	Arlington	Nocardia amarae	+ [a]
Wisconsin	Milwaukee	Nocardia rhodochrous	

[a] No supernatant back in system.

[b] Supernatant returned into the system.

[c] Supernatant returned after heavy chlorination.

Table 2. SOURCES OF STRAINS USED IN THIS STUDY

Strain designation[a]	Identity	Source
SN 5101	Nocardia rhodochrous[b]	R. Bonicke
SN 5104	Nocardia rhodochrous[b]	R. Bonicke
A 12974	Nocardia rhodochrous[c]	R. E. Gordon
I 3639	Nocardia rhodochrous[c]	R. E. Gordon
I 1240	Nocardia rhodochrous[c]	R. E. Gordon
NC 8139	Nocardia rhodochrous[c]	R. E. Gordon
I 1082S	Nocardia rhodochrous[c]	R. E. Gordon
I 624	Nocardia rhodochrous[c]	R. E. Gordon
I 369	Nocardia rhodochrous[c]	R. E. Gordon
I 462	Nocardia rhodochrous[c]	R. E. Gordon
I 515	Nocardia rhodochrous[c]	R. E. Gordon
I 570	Nocardia rhodochrous[c]	R. E. Gordon
I 625	Nocardia rhodochrous[c]	R. E. Gordon
N 10	Nocardia rhodochrous	M. Goodfellow
N 31	Nocardia rhodochrous	M. Goodfellow
N 60	Nocardia rhodochrous	M. Goodfellow
N 239	Nocardia rhodochrous	M. Goodfellow
NC 1621	Strain related to N. rhodochrous[d]	R. E. Gordon
A 7005	Strain related to N. rhodochrous[d]	R. E. Gordon
A 11890	Strain related to N. rhodochrous[d]	R. E. Gordon
I 384	Strain related to N. rhodochrous[d]	R. E. Gordon
I 549	Strain related to N. rhodochrous[d]	R. E. Gordon
I 564	Strain related to N. rhodochrous[d]	R. E. Gordon
I 1256	Strain related to N. rhodochrous[d]	R. E. Gordon
Mil 14	N. rhodochrous	Abnormal foam, Milwaukee, Wis., R.F. Lewis
Mil 15	N. rhodochrous	Abnormal foam, Milwaukee, Wis., R.F. Lewis
SJ 2	N. rhodochrous	Abnormal foam, San Jose, Calif., R. F. Lewis
Se 113	N. rhodochrous	Abnormal foam, Delran, N. J.
Se 135	N. rhodochrous	Abnormal foam, Middletown, N. J.
Se 141	N. rhodochrous	Abnormal foam, Ocean Township, N. J.
Se 167	N. rhodochrous	Abnormal foam, Hartford, Conn.
Se 187	N. rhodochrous	Abnormal foam, Hartford, Conn.
Se 188	N. rhodochrous	Abnormal foam, Hartford, Conn.
Se 189	N. rhodochrous	Abnormal foam, Bordentown, N. J.
Se 192	N. rhodochrous	Abnormal foam, Bordentown, N. J.
Se 194	N. rhodochrous	Abnormal foam, Bordentown, N. J.
Se 3	Nocardia amarae	Abnormal foam, Andover (Miami, Fla.
Se 6	Nocardia amarae	Abnormal foam, Andover (Miami), Fla.
Se 45	Nocardia amarae	Abnormal foam, Andover (Miami), Fla.
Se 51	Nocardia amarae	Abnormal foam, Riverdale (Miami), Fla.
Se 53	Nocardia amarae	Abnormal foam, Riverdale (Miami), Fla.
Se 61	Nocardia amarae	Abnormal foam, Ocean Township, N. J.
Se 64	Nocardia amarae	Abnormal foam, Ocean Township, N. J.

Table 2 (continued). SOURCES OF STRAINS USED IN THIS STUDY

Strain designation[a]	Identity	Source
Se 65	Nocardia amarae	Abnormal foam, Ocean Township, N. J.
Se 85	Nocardia amarae	Abnormal foam, Austin, Tex.
Se 87	Nocardia amarae	"Normal" froth, Austin, Tex.
Se 90	Nocardia amarae	Abnormal foam, Austin, Tex.
Se 91	Nocardia amarae	Abnormal foam, Austin, Tex.
Se 96	Nocardia amarae	Sludge return, Austin, Tex.
Se 97	Nocardia amarae	Abnormal foam, Bordentown Township, N. J.
Se 102	Nocardia amarae	Abnormal foam, Bordentown Township, N. J.
Se 106	Nocardia amarae	Abnormal foam, Eversham, N. J.
Se 107	Nocardia amarae	Abnormal foam, Eversham, N. J.
Se 110	Nocardia amarae	Abnormal foam, Delran, N. J.
Se 111	Nocardia amarae	Abnormal foam, Delran, N. J.
Se 117	Nocardia amarae	Abnormal foam, Matawan, N. J.
Se 118	Nocardia amarae	Abnormal foam, Matawan, N. J.
Se 119	Nocardia amarae	Abnormal foam, Matawan, N. J.
Se 120	Nocardia amarae	Abnormal foam, Matawan, N. J.
Se 122	Nocardia amarae	Artificial foam lab isolate, Bordentown, N. J.
Se 139	Nocardia amarae	Abnormal foam, Ocean Township, N. J.
Se 144	Nocardia amarae	Abnormal foam, Ocean Township, N. J.
Se 149	Nocardia amarae	Abnormal foam, Jamaica Bay, N. Y.
Se 149B	Nocardia amarae	Abnormal foam, Jamaica Bay, N. Y.
Se 151	Nocardia amarae	Abnormal foam, Jamaica Bay, N. Y.
Se 154	Nocardia amarae	Abnormal foam, Jamaica Bay, N. Y.
Se 156	Nocardia amarae	Sample No. 1, abnormal foam, Arlington, Va.
Se 157	Nocardia amarae	Sample No. 1, abnormal foam, Arlington, Va.
Se 160	Nocardia amarae	Sample No. 1, abnormal foam, Arlington, Va.
Se 162	Nocardia amarae	Sample No. 2, abnormal foam, Arlington, Va.
Se 164	Nocardia amarae	Sample No. 2, abnormal foam, Arlington, Va.
Se 18	Nocardia asteroides	Abnormal foam, Andover Plant, Miami, Fla.
Se 43	Nocardia asteroides	Abnormal foam, Andover Plant, Miami, Fla.
Se 73	Nocardia asteroides	Abnormal foam, East Windsor, N. J.
Se 75	Nocardia asteroides	Abnormal foam, East Windsor, N. J.
Se 93	Nocardia asteroides	Abnormal foam, Austin, Texas
Se 114	Nocardia asteroides	Abnormal foam, Delran, N. J.
Se 205	Nocardia asteroides	Abnormal foam, Bedminster Inn, Bedminster, NJ
Se 72	Nocardia caviae	Abnormal foam, East Windsor, N. J.
Se 81	Nocardia caviae	Abnormal foam, East Windsor, N. J.
Se 83	Nocardia caviae	Abnormal foam, East Windsor, N. J.

[a] American Type Culture Collection, Rockville, Md.: I = Institute of Microbiology, Rutgers University; NC = National Collection of Type Cultures, London, England; N = Collection of the University of Newcastle Upon Tyne (England).

[b] Received as N. pellegrini.

[c] Received as "Mycobacterium" rhodochrous.

[d] Received as related to "Mycobacterium" rhodochrous.

Table 3. A COMPARISON OF THE PHYSIOLOGICAL CHARACTERISTICS OF N. AMARAE WITH OTHER SPECIES OF NOCARDIA

Determination	N. amarae (35 strains)	N. asteroides (137 strains[a])	N. brasiliensis (62 strains[a])	N. caviae (24 strains[a])
Hydrolysis of:				
Casein	0[b]	0	98	0
Hypoxanthine	0	4	94	100
Tyrosine	0	1	100	0
Xanthine	0	0	0	100
Adenine	0	0	3	4
Starch	86(w)	67	55	54
Gelatin	0	34[c]		
Esculin	100	100	100	100
Urea	100	96	100	92
Production of:				
Nitrate reductase	100	86	90	100
Growth at/on/in:				
10 C (YD)	6	15	37	13
45 C (YD)	0	41	2	50
Lysozyme broth	9	100	100	100
Acid from:				
Adonitol	0	0	0	0
Arabinose	0	0	0	4
Dulcitol	0	0	0	0
Erythritol	0	7	2	0
Galactose	0	27	94	0
Glucose	100	98	97	100
Glycerol	100	99[d]	98[d]	100[d]
Inositol	92	3	100	100
Lactose	0	0	0	0
Maltose	100	6	4	18
Mannitol	100	1	94	90
Mannose	100	17	68	36
Melibiose	0			0
α-Methyl-D-glucoside	0	0	0	0
Raffinose	0	0	0	0
Rhamnose	92	32	0	5
Salicin	100	25[c]		
Sorbitol	0	0	0	0
Xylose	0	0	0	5

Table 3 (continued). A COMPARISON OF THE PHYSIOLOGICAL CHARACTERISTICS OF *N. AMARAE* WITH OTHER SPECIES OF *NOCARDIA*

Determination	*N. amarae* (35 strains)	*N. asteroides* (137 strains[a])	*N. brasiliensis* (62 strains[a])	*N. caviae* (24 strains[a])
Utilization of:				
Acetate	100	100[d]	100[d]	100[d]
Benzoate	0	0[d]	2[d]	0[d]
Citrate	0	38	98	29
Lactate	100	31[e]		
Malate	100	97	100	100
Oxalate	0	0[e]		
Propionate	100	100[d]	100[d]	100[d]
Pyruvate	100	99[d]	100[d]	100[d]
Succinate	100	92	100	100
Survival:				
50 C/8 h	0	94	0	88

[a] Gordon and Horan (1968).

[b] Percent positive; w, weak.

[c] Gordon and Mihm (1957); (79 strains of *B. asteroides*).

[d] Gordon and Mihm (1962); (142 strains of *N. asteroides*; 15 strains of *N. caviae*; 62 strains of *N. brasiliensis*).

[e] R. E. Gordon, personal communication.

Table 4. A COMPARISON OF THE PHYSIOLOGICAL CHARACTERISTICS OF N. AMARAE WITH OTHER SPECIES OF NOCARDIA

Determination	N. amarae (35 strains)	N. rhodochrous (97 strains)[a]	N. rhodochrous (17 strains)[b]	Nocardia sp. related to N. rhodochrous (36 strains)[a]	Nocardia sp. related to N. rhodochrous (7 strains)[b]	N. rhodochrous (sewage) (12 strains)[b]	N. vaccinii IMRU 3500[b]	N. carnea IMRU 3419[b]
Hydrolysis of:								
Casein	0[c]	0		0		0	-	-
Hypoxanthine	0		0		0	0	-	-
Tyrosine	0	74		5		58	-	-
Xanthine	0	0		0		0	-	-
Adenine	0		35		71	75	-	-
Starch	86(w)[d]	97	35	97	43	25	-	-
Gelatin	0		0		0	0	-	-
Esculin	100		76		86	75	+	+
Urea	100	73[e]			100	83	+	-
Production of:								
Phosphatase	83[d]		100		100	100	-	+
Nitrate reductase	100	82[e]			100	58	+	+
Growth at/in/on:								
10 C (YD)	6[d]	100	100	97	100	94	-	+
45 C (YD)	0	46	53	11	14	66	-	-
Lysozyme broth	9[d]		59		28	94	+	+
5% NaCl (YD)	6[d]		100		100	83	-	+
Adenine agar	6[d]		100		100	100	+	+
Acid from:								
Adonitol	0		0		0	0	-	-
Arabinose	0	8		0		0	-	-
Cellobiose	0		6		0	0	-	-
Dulcitol	0	0		0		0	-	-
Erythritol	0		12		0	0	-	-
Fructose	100		100		43	100	+	+
Galactose	0		6		0	17	+	+
Glucose	100	99		100		100	+	+
Glycerol	100		94		57	100	+	+
Inositol	92[d]	21		0		75	+	-
Lactose	0	0		0		0	-	-
Maltose	100	27[e]	35		14	8	-	-

Table 4 (continued). A COMPARISON OF THE PHYSIOLOGICAL CHARACTERISTICS OF N. AMARAE WITH OTHER SPECIES OF NOCARDIA

Determination	N. amarae (35 strains)	N. rhodochrous (97 strains)[a]	N. rhodochrous (17 strains)[b]	Nocardia sp. related to N. rhodochrous (36 strains)[a]	Nocardia sp. related to N. rhodochrous (7 strains)[b]	N. rhodochrous (sewage) (12 strains)[b]	N. vaccinii IMRU 3500[b]	N. carnea IMRU 3419[b]
Acid from:								
Mannitol	100	99	100	0	0	100	+	+
Mannose	100	99		61		100	+	-
Melibiose	0		0		0	0	-	-
α-Methyl-D-glucoside	0	0		0		0	-	-
Raffinose	0	0		0		0	-	-
Rhamnose	92[d]	9		0		0	+	-
Salicin	100		29		0	25	-	-
Sorbitol	0	100	100	0	0	100	-	+
Sucrose	100		100		29	100	-	-
Trehalose	100	97		24		92	+	+
Xylose	0		41		0	42	+	-
β-Methyl-D-xyloside	0		0		0	0	-	-
Utilization of:								
Acetate	100		100		100	100	+	+
Benzoate	0	70		19		83	-	-
Citrate	0	90		8		100	+	-
Lactate	100	100		100		100	+	-
Malate	100	99		94		100	+	+
Oxalate	0	0		0		0	-	-
Propionate	100		100		100	100	+	+
Pyruvate	100		100		100	100	+	+
Succinate	100	100		97		100	+	+
Tartrate	0		12		0	0	-	-
Survival:								
50 C/8 h	0		82		86	92	ND[f]	ND

[a] R. E. Gordon (1966).

[b] Run by authors.

[c] Percent positive; w, weak.

[d] Reactions of type strain (ATCC 27808); starch hydrolysis -; phosphatase production +; growth at 10 C -; growth in/on: lysozyme broth-, 5% NaCl (YD) - adenine agar -; acid from: inositol +, rhamnose +.

[e] Based on tests of 60 strains (Gordon and Mihm, 1957).

[f] ND = Not determined.

M. rhodochrous) are: nocardomycolic acid type, hydrolysis of adenine, growth at 10 C, growth on adenine and 5% NaCl, acid from fructose, maltose, mannitol, rhamnose, salicin, sucrose, and trehalose, and survival at 50 C.

The new organism also differs from the Russian N. ucrainica (Krasnikov et al. [12]) by eleven characters: acid from galactose, lactose, α-methyl-d-glycoside, raffinose, rhamnose, and xylose, utilization of citrate, decomposition of tyrosine and casein, and growth on 5% NaCl.

Dr. Ruth E. Gordon of our Institute informed us (personal communication, 1973) that she had no named strains of Nocardia in the IMRU collection having the same pattern of physiological reactions as our organism.

On the basis of the overall evidence, we felt these organisms represented a new species; thus we have proposed the name Nocardia amárae, to accommodate this new group (Lechevalier, M. P. and Lechevalier, H. A. [17]).

All of the strains of the new organism had a cell wall of type IV (Lechevalier, H. A. and M. P. Lechevalier [14]) and a whole-cell sugar pattern of type A (Lechevalier, M. P. [15]) typical of members of the genus Nocardia. They contained nocardomycolic acids (Lechevalier, M. P. et al. [16]) of a novel type whose main α branch is mono-unsaturated. Thus, the mycolates of these strains give rise, on pyrolysis, to major amounts of mono-unsaturated fatty esters having 16 and 18 carbons, accompanied for some strains by minor amounts of analogous saturated fragments. This is in contrast to most Nocardia strains we have examined to date which contain nocardomycolates whose pyrolysis fragments are saturated fatty esters having 12 to 18 carbons. Nevertheless, minor amounts of unsaturated fragments have been noted in the pyrolysates of the mycolic acids of numerous nocardiae, particularly members of the N. rhodochrous group (Lechevalier, M. P., unpublished results). We have found this novel type of nocardomycolate in two other nocardial strains: N. vaccinii IMRU 3500 and N. carnea 3419.

As indicated in Table 1, not all actinomycetes isolated from sewage foams were strains of N. amarae. Some of the organisms (Table 2) had the properties of N. caviae and N. asteroides (Table 3). Strains of mycobacteria could also be isolated with great regularity from foams. These organisms, because of their high lipid content, tend

to float and are probably entrapped in the network of the nocardial hyphae. No work has been carried out to determine the taxonomic status of these mycobacterial strains. It is conceivable, however, that they may be a potential public health hazard. In the case of the small package plant serving Bedminster Inn, strains of Streptomyces, Micromonospora and Actinomadura were isolated in addition to strains of N. asteroides. One could probably safely assume that in that plant the percentage of soil contamination is minimal and that of human contributions are maximal.

In summary, we have noted that the well-branched actinomycete most often found associated with sewage foam is a new species of Nocardia which we have named N. amarae. The next most common species is N. rhodochrous. Strains of the pathogenic N. asteroides and N. caviae are next in importance and the least frequently encountered actinomycetes are strains of Streptomyces, Micromonospora and Actinomadura.

Antibiotic activity of Nocardia amarae

The antibiotic activity of N. amarae may have a certain ecological importance in sewage-treatment plants since N. amarae may become a dominant organism as a consequence of its antagonistic activity.

Twenty-one strains of N. amarae, 2 of N. caviae and 3 of N. rhodochrous all isolated from sewage foams, were tested by cross-streak test against 4 fungi (Prototheca portoricensis, Candida albicans, Geotrichum sp., Penicillium notatum) and 4 bacteria (Escherichia coli, Pseudomonas aeruginosa, Mycobacterium smegmatis and Staphylococcus aureus) on two media (Yeast extract-Czapek's, Nutrient glucose agar). Plates were incubated at 28 C and zones of inhibition were observed at 24 hr for bacteria, 72 hr for the Mycobacterium and 96 hr for the fungi.

The strains of N. caviae and N. rhodochrous exhibited no antibiotic activity against any of the test organisms. Twenty strains of N. amarae were active against C. albicans, 1 against P. notatum, 14 against M. smegmatis and 5 against S. aureus.

Three strains of N. amarae (Se 110, Se 110-DHMS-R, Se 151) were selected for a more detailed study of antibiotic activity by cross-streak test. The 8 test organisms listed above were used, but four assay media were investigated. Zones of inhibition, when present, were measured after 3, 5 and 9 days of incubation at 28 C. Streptomyces fradiae strain 3535 known to produce both neomycin

and fradicin was used as a positive control. The 4 assay media used were Yeast extract-Czapek's (YCZ), Bennett's Nutrient and Soil extract agars. On all media, S. fradiae inhibited all the test organisms, and at times, the inhibition was total.

The 3 strains of N. amarae never showed antibiotic activity against the 2 gram-negative bacteria, but some antibiotic activity could be demonstrated against all the other organisms on yeast extract-Czapek's agar. As a rule the zones of inhibition were very narrow (about 2 mm). The best activity was against C. albicans (up to 20 mm) and M. smegmatis (up to 6 mm). There were no obvious differences between the antibiotic activities of the three strains of N. amarae.

Strain Se 110-DHSM-R was grown in liquid Yeast extract-Czapek's medium at 28 C and tested for antibiotic activity after 3, 8 and 14 days of incubation. Antibiotic activity was tested by the streak dilution assay method against C. albicans, M. smegmatis and S. aureus. Both the filtrates of the culture and the homogenate of the disrupted cells were tested for antibiotic activity. No trace of activity was detected in any case.

On the basis of these experiments we could conclude that it is most unlikely that N. amarae gains a predominant position in activated sludge through the production of antibiotics.

Pathogenicity of sewage actinomycetes

Eleven strains of Nocardia isolated from foams were sent to Dr. F. Mariat at the Pasteur Institute in Paris for pathogenicity testing in animals. The identity of the strains was withheld from Dr. Mariat until the end of the test. The strains were grown on Bennett's medium, suspended in physiological saline and ground in a tissue grinder. Inoculation of the suspensions was carried out intra-peritoneally. Guinea pigs received twice the dose given to mice. For each strain a total of eight guinea pigs and eleven mice were inoculated. After one month, surviving animals were sacrificed, examined for lesions, and retrocultures were attempted. The results are summarized in Table 5 and indicate that strains isolated from foam and identified as N. caviae and N. asteroides have a tendency to be pathogenic to laboratory animals whereas N. amarae seems, fortunately, to be innocuous. N. asteroides and N. caviae are opportunistic pathogens and the results obtained by Dr. Mariat are typical of what may be expected with members of these species under the experimental conditions used (Kurup et al. [13]).

Table 5. PATHOGENICITY OF SOME STRAINS OF *NOCARDIA* FROM SEWAGE FOAM

Strain No.	Identity *Nocardia*	Guinea pigs			Mice		
			Sacrificed after 1 month			Sacrificed after 1 month	
		Death[a]	lesions	cultures	Death[a]	lesions	cultures
Se 72	*caviae*	3, 17	-	-	15, 17, 18	+	+
Se 18	*asteroides*		-	-		+	+
Se 93	*asteroides*		-	-		-	-
Se 3	*amarae*		-	-		-	-
Se 64	"		-	-		-	-
Se 85	"		-	-		-	-
Se 91	"		-	-		-	-
Se 102	"		-	-		-	-
Se 107	"		-	-		-	-
Se 111	"		-	-		-	-
Se 117	"		-	-		-	-

[a] Days of death. Dead animals had numerous lesions, cultures were positive.

SECTION V

PRODUCTION OF ACTINOMYCETIC FOAMS IN THE LABORATORY

Introduction

After a number of unsuccessful experiments, it was found that the production of actinomycetic foam in the laboratory was easy to obtain. At no time, however, were we able to obtain foams as thick and as viscous as those produced in affected plants.

In the laboratory, foam was produced by bubbling air through actinomycete-containing activated sludge. Four liter bottles were used containing a liter and a half to two liters of sludge. Moist air was bubbled through a bottom sparger at the rate of 1 to 3 liters per minute. Aeration at 3 liters per minute was better than at 1 liter per minute but excessive foaming often forced a reduction in the rate of aeration. Air was permitted to escape from the aeration bottles after passing through a cotton filter. Good foaming was obtained if the sludge had a dry solid content of 0.5 to 1%. It was possible to keep the foaming going for several days in a given aeration bottle by stopping the aeration daily for one hour, aspirating the clear supernatant and replacing it with raw sewage or with a nutritive solution.

The Bernardsville Sewage-treatment Plant was free of foaming problems until recently (see pg. 48). At the time no foaming was occurring, microscopic examination of its activated sludge (pH usually slightly above 7.0) revealed that it contained no visible actinomycetic hyphae. Bernardsville sewage taken after primary treatment was placed in aeration jars and aerated for more than a month. Every day during this period, the aeration was stopped and after settling, half of the volume of liquid was removed and was replaced by fresh sewage from Bernardsville. Thus a small amount of sludge was eventually formed and this laboratory-made sludge was microscopically free of actinomycetic growth.

In a second experiment, Bernardsville sludge, concentrated to 0.7-0.9% solids by decantation, was inoculated with actinomycetic foam received from Miami. The actinomycete could be maintained in a healthy state (continuing to form foam) by adding fresh raw sewage daily (obtained from the Rutgers University pumping station) after aspiration of part of the contents of the aeration flasks as described above. The same experiment was repeated with foam coming from local sources such as Ocean Township and Bordentown. As a rule during the aeration process, the pH of the sludge dropped to 6.5-5.5.

In addition, aerated sludge from Bernardsville fed with the same raw sewage as in the second experiment, was inoculated with six different pure cultures of actinomycetes (Se 3, Se 61, Se 65, Se 67, Se 74, and Se 75) which had been isolated by plating out foams from various plants. In all cases, foaming was obtained in the laboratory.

We conclude that the combination of raw sewage from Rutgers and sludge from Bernardsville was capable of supporting the growth of actinomycetes with resultant foaming.

Effect of aeration rate on the biomass production and the formation of foam by strains of N. amarae

The growth of N. amarae in Czapek's medium dispensed in baffled and unbaffled 250 ml Ehrlenmeyer flasks was compared. Foam was simply recorded as being present or absent. Foam was observed only in baffled flasks although growth was faster in the unbaffled type (Table 6).

Similar results were observed by growing N. amarae Se 6 in sparger-aerated two liter bottles containing Czapek's medium. After four days of operation, the foam was thicker where the aeration had been the most vigorous although growth was maximal at a lower aeration rate (Table 7). This was due to the fact that in the bottles or flasks where the aeration was greatest many cells were carried away from the growth medium by the foam and deposited on the sides of the bottles where they no longer grew.

Foaming factor

A surface active agent is produced by Nocardia amarae which favors the formation of foam. It was found that in unbaffled flasks of YCZ or low sucrose YCZ (YCZ/5), that the greater the amount of nocardial growth, the greater the amount of the surface activity

Table 6. GROWTH AND FOAM FORMATION OF N. AMARAE STRAIN SE 214 IN BAFFLED AND UNBAFFLED FLASK CONTAINING CZAPEK MEDIUM

Day of incubation	Unbaffled flask		Baffled flask	
	Klett units	Foam formation	Klett units	Foam formation
0	23	-	23	-
1	56	-	45	-
2	125	-	65	-
3	310	-	155	+
4	570	-	450	+
5	700	-	630	+
6	750	-	750	+

Table 7. EFFECT OF AERATION RATE ON THE BIOMASS PRODUCTION AND FOAM OF N. AMARAE STRAIN SE 6 IN CZAPEK MEDIUM AFTER 1 WEEK OF INCUBATION

Aeration rate	Biomass production in 1 week	Foam formation in 4 days
l/min	pcv[a] (ml/liter)	cm[b]
0	1.4	0
1.8	3.8	10
2.4	2.9	24
3.3	2.5	65

[a] Packed cell volume expressed as milliliters per liter broth culture.

[b] Distance between surface of solution and zone of biomass accumulation.

produced (Table 8). Since the increase in amount of the agent parallels the increase in the amount of cells of the nocardia, it is possible that it is a cytoplasmic protein or even a "soap" derived from cell lipids. Also, as illustrated in Table 9, the foam formation is enhanced and stabilized by the presence of the cells, since when these are removed by centrifugation, the foam collapses more readily.

Use of antifoam compounds in the laboratory

Once a method was developed to obtain actinomycetic foam in the laboratory, a few antifoam compounds were tried in order to determine if they had any effect. The following compounds produced by Hodag Chemical Corp. of Skokie, Illinois, were investigated: S-9, S-49, S-118 and PPG-2000. PPG-2000 is a polyglycol; the other compounds are of unknown composition. Foaming was reduced in all cases even at the lowest concentration used (0.1 ml per $1\frac{1}{2}$ liter per day), but the actinomycetic growth was not eliminated. The most promising of these compounds seem to be the polyglycol. However, foaming was not completely eliminated, only reduced, at the highest concentration used (0.4 ml per $1\frac{1}{2}$ liter per day).

In the use of antifoam compounds one should make a distinction between using these compounds as a preventive measure and as a curative measure. Possibly prevention might be the most effective method, however cost and potential deleterious effect on the treatment of the sewage would have to be estimated.

Effect of addition of calcium carbonate on foaming

It had been observed that foam samples collected in a sewage-treatment plant always have a pH lower than that of corresponding sewage. In addition, it was noted that aeration of sewage and activated sludge in the laboratory produced a lowering of the pH. Powdered calcium carbonate, at the level of 10 and 20 grams per liter, was added to Bernardsville activated sludge inoculated with Bordentown foam. Foaming was abundant in all flasks and all showed good growth of the actinomycetes. The addition of 10 gm per liter of calcium carbonate did not affect the pH. Doubling the dose permitted a differential of one pH unit between treated flasks and the control (5.8 vs. 6.8). When the aeration was stopped, sedimentation of sludge was more rapid in calcium carbonate-containing flasks than in the untreated control.

Table 8. FOAM[a] FORMATION BY N. AMARAE SE 110 IN YCZ AND YCZ/5 IN UNBAFFLED FLASKS

Day	YCZ Medium			YCZ/5 Medium		
	pcv[b] (ml/liter)	mm Foam[a]		pcv (ml/liter)	mm Foam[a]	
		0 min	60 min		0 min	60 min
1	20	5	4	20	7	6
2	120	13	13	40	9	9
3	80	14	12	36	10	10
4	84	14	12	30	10	10
5	80	12	12	30	10	10
Uninoculated broth	-	7	0	-	7	0

[a] Foam formation in mm produced on 5 cc of whole broth (cells + medium) mixed on vibromix for 30 secs. in standard 16 x 150 mm tubes, measured immediately and after 60 min. standing.

[b] pcv = packed cell volume.

Table 9. 5 DAY OLD BROTH CULTURE OF N. AMARAE SE 110 GROWN IN UNBAFFLED FLASKS OF YCZ MEDIUM (220 RPM)

	mm of Foam[a]		
	Time after vibromixing		
	0 min	1 min	60 min
Control medium (uninoculated)	7	3	0
Broth culture (cells present)	12	12	12
Broth culture (cells removed by centrifugation at 12,800 G/10 min)	14	11	8

[a] See Table 8.

The practice of maintaining a near-neutral pH by addition of calcium carbonate to sewage is undoubtedly beneficial to the growth of N. amarae; since this organism, like most actinomycetes, is inhibited by pH's lower than 5.0. In this case, the changing of the pH of the foam from 5.8 to 6.8 by the use of calcium carbonate did not appear to have any effect on the actinomycete growth or the foam's stability.

SECTION VI

SEARCH FOR BIOLOGICAL INHIBITORS FOR NOCARDIA AMARAE

It seems possible that the activated sludge of plants without actinomycetic foaming problems might be rich in some biological entities which would prevent the proliferation of N. amarae. Conceivably, these biological factors could be nocardiophages, parasitic bacteria of the Bdellovibrio type, antagonistic microorganisms, or microorganisms producing nocardiolytic enzymes or lipases capable of degrading nocardial lipids.

In order to be able to measure the disappearance of N. amarae in a given sludge quantitatively, it is helpful to incorporate a selective inhibitor in the plating out medium in order to prevent the growth of most sewage organisms while still permitting the proliferation of N. amarae.

Twelve strains of N. amarae were tested for sensitivity to 10 antimicrobial agents on Yeast-Czapek's agar. The disk assay method was used with chloramphenicol, polymyxin B, triple sulfa, neomycin, dihydrostreptomycin, erythromycin, penicillin, tetracycline, bacitracin and streptomycin. All strains were resistant to triple sulfa but were sensitive to the other antimicrobial agents. Since the antimicrobial range of activity of triple sulfa is not wide enough to be of use in repressing the sewage flora during the plating out procedure, a highly dehydrostreptomycin-resistant mutant (resistant to at least 400 μg/ml) was developed from one of the strains of N. amarae (Se 110). This strain (Se 110-DHSM-R) can be added to activated sludge from a non-foaming plant and its fate can be followed quantitatively by plating out on a dihydrostreptomycin-containing medium.

a) Study of an antagonist from the sludge

Washed cells of N. amarae Se 110-DHSM-R were placed in a salt solution fortified with vitamins to which was added some Bernardsville sludge which has never been known to foam. Under such conditions, sewage organisms had only cells of N. amarae as sources of carbon and nitrogen. Flasks were incubated on a rotary shaker at 28 C. At the start of the experiment, and after 4 and 10 days, counts of N. amarae were made on a medium containing 400 µg/ml of dihydrostreptomycin. Other bacteria were counted on antibiotic-less medium. During the incubation period, a reduction in the viable population of N. amarae was observed and the establishment of a dominant bacterium was noted.

This bacterium (No. 3 isolate) was isolated and tested for antibiotic activity by cross streak test against a number of nocardiae, mycobacteria, bacteria and fungi. It was especially very active against strains of Pseudomonas and inactive against fungi. The properties of the bacterium were investigated (Table 10) and the organism was identified as an atypical strain of Enterobacter aerogenes.

In liquid shake cultures, bacterium No. 3 failed to produce any antibiotic. An investigation of the differences observed between the antibiotic activity as shown by cross-streak test and the lack of activity in liquid media revealed that the activity of the bacterium on solid media was due to the production of acid(s). When the growth medium was neutralized with solid $CaCO_3$, no activity was noted. The nature of this acid was not determined since it was felt that in the sludge, which is maintained close to neutrality in most plants, bacteria, such as the No. 3 isolate, could not play a role in the control of the nocardiae.

b) Attempt at isolating nocardiolytic organisms from sludge

Direct plating

Fresh sludge samples obtained from Bernardsville and Middletown, N. J. plants (neither of which showed foaming) were diluted and plated out on yeast extract-peptone or vitamin salts medium containing washed cells of N. amarae Se 110. After incubation at 28° for 2, 6 and 10 days, the plates were examined for clearing of the amarae cells. No lytic microorganisms were recovered.

Table 10. MORPHOLOGICAL AND BIOCHEMICAL PROPERTIES OF #3 ISOLATE

Properties	Results
Gram stain[a] and morphology	Gram-negative rods with rounded ends occur mostly single, some in pairs; occasionally very long wavy rods not clearly consisting of single cells.
5% sheep blood agar	No hemolysis, diameter 2-3 mm
EMB agar	Metallic sheen, diameter 2 mm
MacConkey agar	Red mucoid colony, diameter 2 mm
Blood Phenylethyl alcohol agar	No growth
Oxidase[b]	-
Catalase[c]	+
Motility[d]	-
Nitrate to nitrite	+
Triple Sugar Iron Agar	A/Ag.[e] No H_2S.
Lysine-Iron Agar	K/Kg.[e] No H_2S.
Phenylalanine deaminase	-
Indole[f]	-
M. R.[g]	-
V. P.[g]	+
Simmon's citrate	+
Lysine decarboxylase[h]	+
Ornithine decarboxylase[h]	+
Arginine dihydrolase[h]	-
Urease[i]	+
Esculin	+
OF-glucose[j]	F
OF-xylose	Ag
OF-inositol	Ag
OF-glycerol	Ag
OF-dulcitol	-

Table 10 (continued). MORPHOLOGICAL AND BIOCHEMICAL PROPERTIES OF #3 ISOLATE

Properties	Results
OF-cellobiose	Ag
OF-sorbitol	Ag
CTA-glucose[k]	Ag
CTA-maltose	Ag
Purple Broth rhamnose	Ag
Purple Broth raffinose	Ag
YCZ agar containing 400 μg/ml DHSM	No growth.

[a] 18 hrs. YCZ agar slant.

[b] Kovacs method, reagent 1% tetramethyl-p-phenylenediamine aqueous solution.

[c] 24 hrs. nutrient agar slope. 10% H_2O_2.

[d] Young broth culture, wet mount.

[e] A = Acid; K = alkaline; g = gas.

[f] SIM medium, 48 hrs. 37 C.

[g] Culture incubated at room temperature (25 C) up to 3 days V. P. O'Meara's method.

[h] Moeller method.

[i] Christensen's urea medium. Weak reaction compared to Proteus vulgaris.

[j] = OF = Oxidation/Reduction Medium.

[k] = Cystine-Trypic Medium.

Soil was also examined for antagonists toward N. amarae. In this experiment dilutions of garden soil were incorporated into Woodruff's agar along with washed log phase cells of N. amarae Se 110 or Se 149B and incubated for 3 weeks at 24° C. Most colonies which showed antagonistic properties toward the N. amarae strains were Streptomyces; one was a bacterium. Since it is rare to encounter Streptomyces in sewage, and since they probably do not survive long in that environment, it was felt that further work with the bacterium (100-9) might prove more fruitful.

Enrichment techniques

Flasks containing washed cells of N. amarae Se 110-DHSM-R suspended in a vitamin-salts solution and to which Bernardsville sludge had been added were incubated on a shaking machine at 28 C for up to two months. Periodically, the contents of one flask was centrifuged and the supernatant filtered through a Seitz filter. Drops of the filtrate were deposited on lawns of N. amarae in order to detect nocardiophages. Thus far none have been detected.

In other experiments washed log phase cells of N. amarae Se 110 were added to flasks of liquid yeast-extract peptone or vitamin-salts medium along with either untreated or Millipore-filtered sludge from Bernardsville, Enfield or Middletown. After shaking 4-5 days at 220 RPM, and after every subsequent 5 day period, further fresh N. amarae cells were added and shaking continued 3-4 weeks. Flasks were monitored microscopically for the disappearance of amarae cells. Flasks which showed rapid disappearance of the cells were plated out (with and without Millipore filtration) versus a lawn of N. amarae Se 110 or Se 149 in a two-layer solid-semi-solid yeast extract peptone medium and observed for lysed areas after a suitable period of incubation. In this way two different antagonistic bacteria were isolated from Enfield, one from Bernardsville and none from Middletown. A protozoan, Colpoda sp., which caused the disappearance (2-3 days) of amarae in certain flasks was also isolated from both Enfield and Bernardsville sludge. Again no Bdellovibrio-like strains or nocardiophage were found.

Antagonistic activity of bacterial isolates

Sludge isolates

The most rapidly growing and actively lytic bacterial strain (LB4) from sludge (Enfield) was investigated. It is a yellow, gram-negative,

motile, oxidative, catalase-positive, cytochrome oxidase-positive rod. When LB4 was grown on an agar medium such as yeast-peptone (YP) containing cells of N. amarae, microscopic examination after 3 days showed a dissolution of the amarae cells next to the colonies of the bacterium. In shaken liquid culture it was found that:

1. In a medium where good growth of N. amarae (Se 110) and very poor growth of the bacterium takes place, control of the amarae growth occurs only if the bacterium and the actinomycete are inoculated simultaneously (YCZ Medium, Table 11).

2. In a medium where both organisms grow well (Bennett's Table 12) or very poorly (YP, Table 13), the bacterium clumps, then lyses the amarae cells even when added after the growth of amarae has reached stationary phase. The bacterium appears to grow at the expense of the Nocardia.

Inocula for both organisms (LB4 and Se 110) were 24 hr. old yeast-dextrose (YD)-grown cells which were added at 7.5×10^6 cells/flask for N. amarae and 57×10^8 cells/flask for the bacterium. Packed cell volumes were determined by centrifuging 5 cc. of whole broth 3 min. at 1,200 x g.

We conclude that under proper nutritional conditions LB4 stops amarae growth and lyses its cells even when a substantial growing biomass of amarae is already present.

Soil isolate

The bacterium 100-9 antagonistic to N. amarae which was isolated from soil is a yellow, gram-negative non-motile, oxidative cytochrome-oxidase positive rod which produces large amounts of slime on sugar-containing media. The bacterium was tested in YCZ liquid medium vs. N. amarae Se 110. The inoculum of the 24 hr. old, YD-grown bacterial cells was added at 19.6×10^9 cells/flask. N. amarae inocula were like those previously described for the sludge isolate LB4.

In this medium, the bacterium inhibits the growth of N. amarae and causes lysis of log phase but not stationary phase cells (Table 14).

We conclude that addition of these nocardiolytic bacteria to tanks with amarae foams might help control this problem. We were unsuccessful

Table 11. LB4 VS. N. AMARAE SE 110 in YCZ MEDIUM

(POOR GROWTH OF BACTERIUM: GOOD GROWTH OF N. AMARAE)

pcv[a] (ml/liter)

Day	N. amarae Se 110 only	LB4 (bacterium) only	Se 110 + LB4 both added day 0	Se 110 added day 0 + LB4 added day 1	Se 110 added day 0 + LB4 added day 2
1	40.0	6	8	-	-
2	100.0	4	8[b]	100.0[c]	-
3	100.0	4	4[b]	100.0	100.0[c]
4	90.0	4	4[b]	90.0	100.0
6	80.0	2	2[b]	100.0[c]	120.0[c]

[a] pcv = packed cell volume.

Microscopic picture:

[b] No amarae cells.

[c] amarae cells are clumped but healthy looking, and viable.

pH values remained >6.6 in all flasks.

Table 12. LB4 VS. N. AMARAE SE 110 IN BENNETT'S MEDIUM

(GOOD GROWTH OF BOTH STRAINS)

pcv[a] (ml/liter)

Day	N. amarae Se 110 only	LB4 (bacterium) only	Se 110 added day 0 + LB4 added day 0	Se 110 added day 0 + LB4 added day 1	Se 110 added day 0 + LB4 added day 2
1	44.0	36.0	36.0[b]	-	-
2	42.0	28.0	28.0[c]	38.0[d]	-
4	36.0	32.0	44.0[c]	44.0[b]	40.0[d]
5	32.0	36.0	32.0[c]	40.0[b]	40.0[d]
9	40.0	28.0	36.0[c]	44.0[c]	24.0[c]

[a] pcv = packed cell volume.

Microscopic picture:

[b] Lysing amarae cells covered with bacteria.

[c] No amarae cells.

[d] Clumped but healthy-looking amarae cells.

pH's were >6.7 in all flasks.

Table 13. LB4 VS. N. AMARAE SE 110 IN VP MEDIUM

(POOR GROWTH OF BOTH STRAINS)

pcv[a] (ml/liter)

Day	N. amarae Se 110 only	LB4 (bacterium) only	Se 110 added day 0 + LB4 added day 0	Se 110 added day 0 + LB4 added day 1	Se 110 added day 0 + LB4 added day 2
1	12.0	12.0	12.0	-	-
2	14.0	14.0	14.0[b]	14.0[c]	-
3	12.0	4	6.0[d]	10.0[d]	10.0[d]

[a] pcv = packed cell volume.

Microscopic picture:

[b] Lysing amarae.

[c] Clumping but healthy amarae.

[d] Ghosts (empty cells) of amarae.

pH's were >7.0 in all flasks.

Table 14. 100-9 VS. N. AMARAE SE 110 IN YCZ MEDIUM

pcv[a] (ml/liter)

Day	N. amarae Se 110 only	100-9 only	Se 110 added day 0 + 100-9 added day 0	Se 110 added day 0 + 100-9 added day 1	Se 110 added day 0 + 100-9 added day 2
1	50	4	8[b]	-	-
2	120	2	2[c]	20[b]	-
3	84	2	2[c]	4[c]	102[d]
5	78	N	N[c]	N[c]	80[d]

[a] pcv = packed cell volume.

Microscopic picture:

[b] Clumped amarae cells.

[c] No to slight traces amarae cells.

[d] Healthy amarae.

pH's in flasks were >6.0 throughout experiment.

N = Negligible.

in detecting actively lytic nocardiophage and Bdellovibrio-type bacteria active against nocardias. In particular, our repeated failure to isolate nocardial antagonists from the non-foaming Middletown plant makes it seem unlikely that the presence of lytic microorganisms is responsible for the absence of nocardial foams in some sewage treatment plants.

SECTION VII

COMPARISON OF THE OPERATING CONDITIONS

OF FOAMING AND NON-FOAMING PLANTS

The purpose of this study was to compare the operating records of similar wastewater treatment facilities: some having a significant development of actinomycetic growth and the others free of this problem. The aim of this comparison is to find parameters which may have an effect on the actinomycetic growth pattern in wastewater plants.

Wastewater treatment facilities selected for comparison

The wastewater treatment facilities that were selected for comparison in this study are the Ocean Township Sewerage Authority's Wastewater Treatment plant in Ocean Township, Monmouth County, N. J.; the Township of Middletown Sewerage Authority's Wastewater Treatment Plant, in Middletown Township, Monmouth County, N. J. and the Bernardsville Wastewater Treatment Plant in Bernardsville, Somerset County, N. J. In addition, some generalized comparisons were also made utilizing the wastewater treatment plants located at Bordentown Township, N. J., the Madison-Chatham joint meeting plant at Chatham, N. J., and the municipal wastewater treatment plant at Roxbury Township, N. J.

The wastewater treatment facilities utilized in this comparison were all of the activated sludge process type. The treatment facilities at Ocean Township consist of preliminary treatment, primary sedimentation, activated sludge, effluent disinfection, and separate anaerobic sludge digestion. The activated sludge process utilized is of the sludge reaeration type (contact stabilization). Digested sludge is dewatered via vacuum filtration and supernatant from the digesters is oxidized with chlorine in a Purifax unit prior to being returned to the preliminary treatment system. The Ocean Township Treatment facility began operation in October, 1968.

The Middletown Waste Treatment Facility began operation in July, 1971. This facility provides for preliminary treatment, primary sedimentation, activated sludge, effluent disinfection, and separate anaerobic digestion. The activated sludge system has been designed for stepped aeration, however, conventional and sludge reaeration can also be utilized. At the present time the conventional activated sludge system is being employed at this facility. Digested sludge is removed from the facilities via barging with ocean disposal. Supernatant from the digestion system is returned to the preliminary treatment facilities.

The Bernardsville Wastewater Treatment Facility was constructed in 1933. The facilities at the plant consist of preliminary treatment, primary sedimentation, activated sludge, effluent disinfection and anaerobic sludge digestion. The conventional activated sludge process is utilized at this waste treatment facility. Digested sludge is disposed via a dewatering centrifuge and supernatant is returned to the primary treatment system.

Table 15 presents a comparison of the major aspects of these three wastewater treatment facilities.

Actinomycetic problems

The treatment facility at Ocean Township has had an annual actinomycetic foaming problem in the aeration tanks and final settling tanks since 1970. The foam appears in late spring when the sewage temperature rises above 55° F and continues through to the fall, when the sewage temperature drops below 55° F. Various means of controlling the foam with water sprays, defoaming agents, and chlorination have been unsuccessful. In more recent plant control methods, the foam has been reduced to a manageable level by reducing the solids and the dissolved oxygen in the aeration system. It is assumed however, that some reduction of treatment efficiency is also likely with this control method.

The treatment facilities at the Township of Middletown and at Bernardsville, however, have not experienced the actinomycetic foaming problems. Examinations of the activated sludge and scum formed at the Bernardsville plant had not revealed the presence of any actinomycete. Examinations of the Middletown sludge revealed some actinomycete but these were of a very low concentration.

Table 15. TREATMENT FACILITY COMPARISON

Item	Ocean Township	Middletown Township	Bernardsville
Initial operation	October, 1968	July, 1971	1933
Preliminary treatment	Comminutor and grit remover	Mechanical bar screen and grit remover	Comminutor
Primary treatment	Rectangular clarifier	Rectangular clarifier	Rectangular clarifier
Primary sludge and scum disposal	To digester	To digester	To digester
Secondary treatment	Activated sludge sludge-reaeration (contact stabilization)	Activated sludge conventional	Activated sludge conventional
Aeration means	Mechanical turbine	Mechanical turbine	Diffused air
Waste sludge disposal	To primaries	To digester	To digester
Secondary sedimentation	Circular clarifier with scum removal	Circular clarifier with scum removal	Square clarifier
Activated sludge return	Variable speed pumps	Variable speed pumps	Air ejector

Table 15 (continued). TREATMENT FACILITY COMPARISON

Item	Ocean Township	Middletown Township	Bernardsville
Secondary scum disposal	To aeration tanks	To digester	None
Other treatment	Effluent chlorination	Effluent chlorination	Effluent chlorination
Sludge treatment	Anaerobic digesters high rate	Anaerobic digesters high rate	Anaerobic digesters conventional
Supernatant disposal	To wet well after purifax treatment	To wet well	To primaries
Digested sludge disposal	Vacuum filter and land fill	Ocean barging	Centrifuge and land fill

Technical review and comparison of operating records

The operating records covering a two year period for each of the three wastewater treatment plants selected were obtained from the operating agencies and reviewed. Based upon the completeness of the available data and since experience in the New Jersey facilities has indicated that the formation of actinomycetic foam occurred when the temperature of the sewage is above 55° F, the period of April through November, 1972 was selected for detailed review and study.

In evaluating the records of this period, the following observations were made regarding the wastewater characteristics at each treatment facility as detailed in Table 16.

1. The average sewage temperature in the Middletown and Ocean Township Plants were approximately the same and in the range of 63° to 66° F.

2. The pH of the raw sewage for Ocean Township was somewhat lower than the Middletown Township and Bernardsville Sewage Treatment Plants. This was in the realm of a pH difference of 0.1 to 0.2.

3. The BOD of the raw sewage and the BOD of the settled sewage treated by the activated sludge system appeared to be reasonably similar in the Ocean and Middletown Treatment Facilities. These values for the raw BOD ranged between 187 and 217 mg/l and for the settled waste between 123 and 128 mg/l. The Bernardsville values were estimated as 200 and 130 mg/l respectfully.

4. The suspended solids content of the raw sewage and the settled sewage in both the Middletown and Ocean Treatment Plants were again reasonably similar. The raw sewage suspended solids for the plants ranged between 161 and 247 and the settled sewage suspended solids ranged between 107 and 141 mg/l. It is to be noted that the higher values were found in the Middletown Township Plant and are believed due to the influence of the returned supernatant from the anaerobic digestion system.

In all three plants the wastewater received and treated at these facilities was essentially domestic waste with very minor amounts of industrial and/or commercial wastes.

Table 16. SEWAGE CHARACTERISTICS

April to November, 1972

Item	Ocean Township	Middletown Township	Bernardsville
General classification	Domestic	Domestic	Domestic
Industrial and commercial	Minor	Minor	Minor
Average daily flow	2.89 mgd	3.94 mgd	0.44 mgd
Biochemical oxygen demanding (BOD) raw	187 mg/l	217 mg/l	200 mg/l E[a]
To aeration	123 mg/l	128 mg/l	130 mg/l E
Suspend solids raw	161 mg/l	247 mg/l[b]	-
To aeration	107 mg/l	141 mg/l	
Temperature	66° F	63° F	-
pH	6.9	7.1	6.9+

[a] E = Estimated.

[b] Possible excessive supernatant load.

Further comparison of the three treatment facilities in regard to process, loadings, and operating parameters are presented in Table 17 and can be summarized as follows:

1. The mixed liquor suspended solids maintained in the aeration tanks ranged between approximately 2000 to approximately 3800 mg/l. In the reaeration portion of the Ocean Township facility the suspended solids carried in the mixed liquor was approximately 6500 mg/l. A direct comparison with the Ocean Plant, in this aspect, is difficult since the plant is separated into a mixing tank and a stabilization tank with two separate concentrations of mixed liquor suspended solids.

2. The dissolved oxygen maintained in the mixed liquor within the aeration tanks for the Ocean and Middletown Treatment Plants ranged between 2.3 and 2.6 mg/l. Although no information is available as to the dissolved oxygen in the Bernardsville mixed liquor it is believed that this figure would be very low.

3. The rate of return activated sludge utilized in the Ocean Township and Middletown Treatment facilities is approximately the same in ranges between 41 and 47%. The rate of return at the Bernardsville facility is estimated to be 25%.

4. The organic loadings applied to the aeration tanks in the Ocean and Middletown Plants were 14 and 10 lbs. BOD per 100 lbs. mixed liquor suspended solids, respectively, and 30 lbs. BOD per 100 lbs. MLSS in the Bernardsville Plant.

5. The detention times in the final settling tanks at the respective plants were 2.2 hours for Ocean, 3.7 hours for Middletown, and 2.6 for Bernardsville.

6. The sludge age in each of the treatment plants aeration tank were 7.3 days for Ocean, 9.4 days for Middletown, and 3.1 days estimated for Bernardsville.

In regard to a comparison of the physical plants (Table 15), each of the three treatment plants has preliminary treatment and primary sedimentation prior to the activated sludge system. The Middletown and Bernardsville Treatment Plants operate a conventional activated sludge system, whereas the Ocean Township Treatment Plant operates under a sludge reaeration or contact stabilization activated sludge system. The waste sludge from the

Table 17. COMPARISON OF PROCESS, LOADING, AND OPERATING PARAMETERS

April to November 1972

Item	Ocean Township	Middletown Township	Bernardsville
Primary tank-detention time	1.6 hrs	2.4 hrs	2.4 hrs
Aeration tank detention time at Q	3.9 hrs	8.4 hrs	5.1 hrs
Activated sludge return rate	47%	41%	25% E[a]
Mixed liquor suspended solids	Mix. 2160 mg/l Stab. 6535 mg/l	3770 mg/l	1900 mg/l E
Mixed liquor volatile suspended solids		2620 mg/l	
Mixed liquor dissolved oxygen	Mix. 3.5 mg/l Stab. 2.3 mg/l	2.6 mg/l	Very low E
Loading #BOD/#MLSS	0.14	0.10	0.30 E
#BOD/#MLVSS		0.14	

Table 17 (continued). COMPARISON OF PROCESS, LOADING, AND OPERATING PARAMETERS

April to November 1972

Item	Ocean Township	Middletown Township	Bernardsville
Sludge age	7.3 days	9.4 days (6.6 days VSS) 12.3 days[b] (8.6 days VSS)	3.1 days E
Secondary sedimentation tank detention time with return	2.2 hrs	3.7 hrs	2.6 hrs E

[a] E = Estimated.

[b] Based on April to August - no excessive supernatant load.

activated sludge system in the case of Ocean Township is returned to the primaries. In the case of Middletown Township, the waste activated is conveyed to the anaerobic digesters. The plants at Ocean and Middletown are both provided with skimmers and scum baffles on the final settling tanks. The scum collected from these units is returned to the aeration tanks in the Ocean Plant; whereas, the scum is directed to the anaerobic digesters in the Middletown Plant. In all three plants, sludge removed from the system is anaerobically digested with supernatant returned to the primary treatment units. In the case of Ocean Township, the supernatant is oxidized with chlorine prior to discharge into the primary units.

Comparison of domestic water supplies serving each area

A comparison of the water supplies that serve each of the treatment plant service areas was considered, in order to determine whether there were any unusual characteristic differences in the supplies which might contribute to the development of the actinomycetic growths. Analysis of the water supplies were obtained from the purveyor serving each of the respective wastewater treatment plant service areas and were evaluated for chemical composition. Table 18 lists the chemical analyses for the three areas.

In general, the evaluation indicates that the water supplies of the Ocean Township, Middletown Township, and Bernardsville areas are similar in chemical composition with the only notable exceptions being a slightly higher nitrate nitrogen content in the Middletown and Bernardsville waters over that of the water supplied to the Ocean Township area. The analysis indicated the Ocean waters to contain a nitrate nitrogen content of approximately 0.6, the Middletown waters 1.29, and the Bernardsville waters 15.5 mg/l. In a similar manner, some differences were noted in the sodium content of the waters; with Ocean Township having an average of 18, Middletown having an average of 10 and Bernardsville having an average of 12 parts per million. The pH of each of the water supplies was 7.7 for Ocean, 7.7 for Middletown and 7.8 for Bernardsville.

Summary and conclusions

The overall evaluation of the operating records and analysis at the treatment plants in Middletown Township, Ocean Township and Bernardsville does not disclose any significant difference in either the raw sewage characteristics, the characteristics of the drinking water supplied to the general area nor the operational

Table 18. CHEMICAL ANALYSIS OF POTABLE WATER SUPPLIES

Substance	Concentration (mg/l)		
	Ocean Township	Middletown Township	Bernardsville
NH_3 - N	<0.05	<0.05	
NO_3 - N	0.60	1.29	15.5
T - P	<0.05	<0.05	
H_g	<0.00052	0.00063	0
As	<0.0025	<0.0025	0.009
Cu	0.006	0.029	0
Pb	<0.0065	0.008	0.01
Se	<0.0023	<0.0023	0
Na	18	9	12
Zn	0.0063	0.0037	0.05
F^-	1.90	1.94	0.11
Cd	0.0011	<0.001	0.002
pH	7.7	7.7	7.8

(Based on analyses in period 1968 to 1973.)

characteristics of the activated sludge processes used at each treatment facility. There are some minor variations between the parameters of loadings and the specific activated sludge process used; however, these do not appear to be significant to the problem. There is also some minor differentiation between the pH of the raw sewage at each of the treatment facilities and a general indication that perhaps the raw sewage at Ocean Township, which is experiencing the actinomycetic growth problem, may be somewhat lower than the Middletown and Bernardsville raw sewage. Again this minor difference is relatively small and is not believed to be significant.

However, one item of difference was noted between the operation at the Ocean Township facility and that at the Middletown and Bernardsville facilities. This operational difference was in the manner of disposal of the anaerobic supernatant for the digester facilities. In the Bernardsville and Middletown facilities, the supernatant is returned to the primary treatment units without any pretreatment. This in turn creates a loading on the units and can be seen in the Middletown plant as a definite darkening of the mixed liquor contents of the aeration tank. In the Ocean Township Plant, the supernatant is either diverted from the treatment facility or is treated with super-chlorination by a "purifax" unit prior to discharging into the primary unit. As such, the material exerts no loading or other effects on the treatment process.

This treatment of anaerobic supernatant at the Ocean plant, in effect, creates a similarity with several other plants observed to have an actinomycetic foaming problem in the New Jersey area as noted in the following:

a) The treatment facilities at Bordentown Township, are of the aerobic type with aerobic digestion rather than anaerobic digestion. This plant is experiencing problems with actinomycetic foaming.

b) Actinomycetic foaming was also observed at the Madison-Chatham Joint Meeting Treatment Plant where supernatant from anaerobic digesters was being conveyed directly to sand drying beds and not being returned to the system.

c) In similar instances, the plants at Roxbury Twp., Matawan, and East Windsor have had actinomycetic foaming and also have no anaerobic digestion units.

In addition, the latest developments at the Bernardsville plant appear to also indicate a possible relationship between the absence of anaerobic digestion and actinomycetic foaming. Recently this plant process was modified and the anaerobic digestion facilities were taken out of service. After a short operating period, some foaming was observed in the aeration tanks. Examination of this foam revealed the presence of actinomycetic growths.

In light of these findings, it appears conceivable that the presence or absence of an anaerobic supernatant may have a bearing on actinomycetic growth being experienced in some activated sludge treatment plants. As can be seen by an examination of Table 1, all the plants with foam problems either have no anaerobic digester or, if they have one, do not return the untreated supernatant into the system.

SECTION VIII

ANTINOCARDIAL ACTIVITY OF ANAEROBIC DIGESTER SUPERNATANT

We attempted to confirm a possible toxic effect of untreated anaerobic digest on the growth of N. amarae.

In the first experiment, fresh, unchlorinated digest (from Middletown, N. J.) was diluted in sterile water and distributed into flasks of YCZ medium. These flasks were inoculated with 24 hr old log phase N. amarae Se 110 cells and harvested periodically to determine their cell content by packed cell volume. As can be seen in Tables 19 and 20:

1. The anaerobic digest was toxic to amarae even at a final dilution of 10^{-5} to 10^{-6}, depending on the level of solids per ml in the anaerobic digest used.

2. Removal of the solids of the anaerobic digest by filtering through a Millipore Filter (0.45 μ pore) removes the toxic effect.

3. Autoclaving the anaerobic digest partly destroys the toxic principle.

As the pH's of the flasks in the first experiment on amarae inhibition by anaerobic digest were quite low (∿ pH 5.7), a second experiment was run using YCZ medium neutralized with excess $CaCO_3$ (Tables 21 and 22). Here again the Middletown digest showed toxicity when diluted to 10^{3-6} thus:

4. The inhibition of N. amarae by anaerobic digest is not caused by lowered pH alone.

A sample of unchlorinated anaerobic digest supernatant from a second plant (Ocean Township, N. J.) was assayed for toxicity to N. amarae

Table 19. TOXICITY OF ANAEROBIC DIGEST (AD)[a] TO *N. AMARAE* SE 110 IN YCZ MEDIUM

Total pcv[b] (ml/liter)

Day	YCZ control (Se 110 only)	YCZ + AD diluted to						
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
1	44	10	10	18	20	28	44	46
2	116	50[c]	40[d]	18[d]	18[e]	20[e]	64[f]	120[f]
3	70	62[c]	66[d]	90[d]	14[e]	16[e]	62[f]	70[f]
4	68	90[c]	80[d]	120[d]	8[e]	30[e]	80[f]	70[f]
5	76	60[c]	110[d]	100[d]	16[e]	16[e]	68[f]	64[f]
6	70	44[c]	60[d]	100[d]	8[e]	16[e]	76[f]	ND

[a] 25 mg solids (dry weight) per ml.

[b] pcv = packed cell volume.

Microscopic picture:

[c] No *N. amarae* (yeasts + bacteria).

[d] 40% *N. amarae* (estimated); 50% yeasts, 10% bacteria.

[e] 90% *N. amarae* (estimated); no yeasts, 10% bacteria.

[f] 100% *N. amarae*.

Table 20. TOXICITY OF AUTOCLAVED AND MILLIPORE FILTERED ANAEROBIC DIGEST[a] (AD) TO N. AMARAE SE 110 IN YCZ MEDIUM

Total pcv[b] (ml/liter)

	Filtered AD		Autoclaved AD		
Day	YCZ control (Se 110 only)	YCZ + 10^{-1} dilution millipore filtered AD	YCZ control (Se 110 only)	YCZ + 10^{-2} dilution autoclaved AD	YCZ + 10^{-3} dilution autoclaved AD
1	60	60	56	10	46
2	150	130	104	86	104
3	90	70	ND	ND	ND
4	84	60	ND	ND	ND

[a] 25 mg solids (dry weight) per ml.

[b] pcv = packed cell volume.

Table 21. TOXICITY OF ANAEROBIC DIGEST (AD)[a] TO GROWTH OF *N. AMARAE* SE 110 IN YCZ MEDIUM NEUTRALIZED WITH EXCESS $CaCO_3$

Total pcv[b] (ml/liter)

Day	YCZ + $CaCO_3$ control (Se 110 only)	YCZ + $CaCO_3$ + AD diluted to						
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
1	16	12	16	16	16	8	16	16
2	40	2	4	20	20	16	20	40
3	44	4	16	20	30	16	12	34
5	60	4	20	18	34	20	8	44
7	52	Neg.	16	4	20	20	20	30
Estimated % *amarae* cells[c]	100%	5%	10%	30%	30%	30%	70%	98%

[a] 25 mg solids (dry weight) per ml.

[b] pcv = packed cell volume.

[c] Other organisms were mostly bacteria.

Table 22. TOXICITY OF ANAEROBIC DIGEST (AD)[a] TO GROWTH OF N. AMARAE SE 110 IN YCZ MEDIUM NEUTRALIZED WITH EXCESS $CaCO_3$

Corrected N. amarae pcv[b] (ml/liter)[c]

Day	YCZ + $CaCO_3$ (Se 110 only)	YCZ + $CaCO_3$ + AD diluted to			
		10^{-5}	10^{-6}	10^{-7}	10^{-8}
1	2	0	0	0	0
2	80	2	40	90	90
3	80	0	26	80	70
4	70	20	68	64	72
5	54	30	76	60	70

[a] Contained 18 mg solids/ml (dry weight).

[b] pcv = packed cell volume.

[c] Corrected by subtracting pcv figures obtained in YCZ + $CaCO_3$ flasks inoculated with AD alone (measuring the natural microbial population of the AD) from the total pcv (amarae + other microorganisms). Corrected figures represent just the growth of amarae.

grown in YCZ + $CaCO_3$ as above. The digest showed toxicity even when diluted to 10^{-7}, thus:

5. The toxicity of anaerobic digest supernatant to N. amarae is not solely confined to one plant.

Attempt at isolation of the toxic principle

Solids from the anaerobic digest were extracted with methanol, chloroform-methanol (2:1), isopropyl acetate or petroleum ether (B. p. 30-40° C). The pH of the mixture was 7.0. The extracts were taken to dryness with nitrogen, dissolved in dimethyl sulfoxide and Seitz-filtered. Added to YCZ liquid medium at a digest dilution equivalent of 10^{-5} (see Table 23 for definition) no extract had toxicity to growth of N. amarae Se 110. However, a chloroform-methanol (2:1) extract of autoclaved anaerobic digest solids, was dried under nitrogen, taken up in chloroform-methanol (2:1). Seitz-filtered and assayed in YCZ versus N. amarae Se 110 at digest dilution equivalents of 2×10^{-1} and 10^{-1}, it showed toxicity (Table 23). Thus:

6. Organic solvent extracts of anaerobic digest solids are not toxic to N. amarae when assayed at a level equivalent to that normally inhibitory when the solids themselves are used; however, an assay of a chloroform-methanol (2:1) extract at higher concentrations shows that the toxic factor can be partially extracted, by organic solvents under certain conditions.

Characterization of the toxic principle of anaerobic digest

The anaerobic digest was added to flasks of liquid YP and dilute YP (YP/25) which contained hyphae of washed log phase N. amarae Se 110 cells and these were shaken at 28° for 5 days. Although the amarae hyphae appeared clumped they did not lyse. Attempts to isolate organisms antagonistic to amarae from these flasks was unsuccessful. Thus,

7. The toxic principle of anaerobic digest does not cause lysis of amarae cells.

8. There do not appear to be organisms antagonistic to N. amarae in anaerobic digest when tested under these conditions.

Table 23. TOXICITY OF A CHLOROFORM-METHANOL EXTRACT OF AUTOCLAVED ANAEROBIC DIGEST SOLIDS TO N. AMARAE SE 110

pcv[a] (ml/liter)

Day	Solvent control for 2×10^{-1}	Solvent control for 10^{-1}	Solvent extract (Digest dilution equivalent)[b] 2×10^{-1}	Solvent extract (Digest dilution equivalent)[b] 10^{-1}
1	16	20	4	8
2	82	100	4	16
3	86	82	4	38
8	82	80	96	98

[a] pcv = packed cell volume.

[b] Digest dilution equivalent: The extract coming from the amount of anaerobic digest solids contained in a given dilution of the whole anaerobic digest supernatant.

In general, it may be concluded that a thermolabile principle toxic to Nocardia amarae is associated with the solids from anaerobic digestion and that this principle is: 1) poorly soluble in water, 2) soluble in organic solvents under certain conditions, and 3) capable of being diluted to 10^{-5} or 10^{-7} and still show toxic effects (dilution depends on the amount of solids present in the original anaerobic digest).

SECTION IX

DISCUSSION

As we conclude this study, our final hypothesis is that nocardial foams might be prevented by returning untreated anaerobic digester supernatant into the system. This hypothesis is supported by the following evidence: 1) as far as we know nocardial foaming occurs only in plants which either do not have an anaerobic digester or in plants having such facilities in which the supernatant is either not returned or is returned only after some form of treatment such as Purifax chlorination. 2) Samples from the supernatants of anaerobic digesters from two different plants strongly inhibited the in vitro growth of Nocardia amarae, the most common sewage nocardia.

In general, this hypothesis makes sense from an historical point of view. It is only recently that nocardial foams have been noticed by plant operators in spite of the fact that the activated sludge process has been known since 1914 (Anonymous, 1967). However, the classical method of activated sludge treatment has included the return of the supernatant from the anaerobic digesters into the system and recently the trend has been to build more and more plants without anaerobic digesters, to eliminate the digesters from those that have them or to treat the supernatant before returning it into the system in order to reduce the loading factor.

According to our hypothesis, it is thus not surprising that more and more plants report nocardial foaming.

If one reviews the literature on the microbiology of activated sludge, one is struck by the lack of attention paid to actinomycetes (Farquhar and Boyle[3]; van Veen[18]). We can predict that if action is not taken to control nocardial foams, the actinomycetes will become a matter of great concern to sanitation microbiologists.

Logically the next steps in this study are, 1) the testing of our hypothesis in cooperating plants and 2) the elucidation of the nature of the nocardiotoxic compound. This last part of the study will be especially important to operators of plants without anaerobic digesters. If the nature of the toxic principle is known and if the cost does not turn out to be prohibitive, one could conceivably add it to the sewage of plants without anaerobic digesters.

SECTION X

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SECTION XI

LIST OF INVENTIONS

Lechevalier, M. P., and Lechevalier, H. A. Nocardia amarae sp. nov., an actinomycete common in foaming activated sludge. Intern. J. Systematic Bacteriol. 24: 278-288, 1974.

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16. ABSTRACT

In some sewage-treatment plants of the activated sludge type, a thick foam may be formed at the surface of the secondary aeration and settling tanks. Such foams have often been found to be rich in actinomycetes. This report covers the work done on this problem between April 1971 and May 1974. Over 250 strains of actinomycetes have been isolated from foams or activated sludge from 19 different sewage-treatment plants located in 8 states. The actinomycete most commonly associated with foams is a previously undescribed Nocardia which has been given the name N. amarae. It has been demonstrated experimentally in the laboratory that N. amarae may cause the kind of foam observed in the plants. Factors affecting the growth of N. amarae have been studied and a method of control of the foam by addition of digester supernatant to the activated sludge is proposed.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
*Actinomycetales, *Nocardia, Activated sludge process, Foam--cellular materials, Microorganism control (sewage), *Aeration tanks	Digester supernatant	13B

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