COMPUTER ASSISTED IDENTIFICATION OF FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE SYSTEMS

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CHAPTER I

INTRODUCTION

General Statement of the Problem

This thesis describes a PC based expert system program to assist in the identification of different filamentous bacteria that occur in activated sludge wastewater treatment plants. The objective of the thesis was to develop an expert system software, for the personal computer, which can be used to assist in typing of different filamentous bacteria and as a training tool for wastewater treatment plant personnel.

An expert system is an intelligent computer program that uses knowledge and inference procedures to solve problems (Gudavalli, 1987). It has previously been successfully used in the identification application (Barr et al., 1989). What the expert system does is to try to mimic the human brain electronically. In the expert system there is a knowledge base. The computing technique provides a searching scheme called an inference engine. First the user of the computer provides information about a certain problem. Then the expert system searches the knowledge base by using the inference engine to give a result.

In biological wastewater treatment processes, there exist twenty-nine commonly observed filamentous bacteria (Jenkins et al., 1993). Filamentous bacteria are very important to the biological process that occurs in a wastewater treatment plant. They form a backbone for activated sludge floc which helps the sludge settle in the secondary clarifier. However, excessive filamentous bacteria can cause bulking and foaming in the biological process (Sezgin et al., 1978). Research has indicated that various bulking and foaming problems are caused by the type and amount of filamentous bacteria present (Jenkins et al., 1993).

Therefore, correctly identifying filamentous bacteria in activated sludge is very important for proper diagnosis of a specific bulking or foaming problem. It is the necessary first step in bulking and foaming control. Different filaments are associated with different operational conditions (Richard, 1989). These bacteria can be, for instance, sulfur oxidizing filaments, low dissolved oxygen filaments, or low food to microorganism ratio (F/M) filaments. Different filaments require different control methods (Jenkins et al., 1993). Correct typing of filamentous bacteria gives the operator additional knowledge with which to design the proper control for bulking and foaming in the wastewater treatment plant.

Typing of the filamentous bacteria is conducted using a microscope. Under the microscope, each filamentous organism is characterized using a number of parameters. These parameters include branching, motility, filament shape, filament location, attached growth, sheath, crosswalls, filament diameter, filament length, cell shape, cell size, presence of sulfur deposits, presence of other granules, gram stain, and Neisser stain. In

total, 15 parameters were identified by Jenkins et al. (1993). Then, the filaments are identified by matching the observed filament characteristics with the short descriptions provided in the *Manual on the Causes and Control of Activated Sludge Bulking and Foaming* (Jenkins et al., 1993).

Based on information from Eikelboom and van Buijsen (1981), Jenkins et al. provide a short description for each filamentous organism commonly observed in activated sludge in their *Manual on the Causes and Control of Activated Sludge Bulking and Foaming* (Jenkins et al., 1993). There are 29 filamentous organisms described in this manual. The procedure described in this manual to identify each filamentous organism in a wastewater sample is widely used by wastewater treatment personnel.

The key point in identifying a filamentous organism is to match the unique set of 15 characteristics with one of the 29 filaments provided in Jenkins et al. (1993) manual. Among the 29 filaments, many of them have similar characteristics. Some of the filaments are differentiated only by a few of the parameters described above. Therefore, the matching process requires experience. For an experienced observer, it normally takes about two hours to type one sample (Richard, 1989). Also, among wastewater treatment plant operators and environmental engineers, there are only a few who know how to type filaments correctly. Therefore, a tool to assist and speed up the matching process of filamentous bacteria identification would be very important for typing observers.

Why an Expert System Program?

The entire benefit of an expert system is its ability to model human thought in a manner that can be processed on a computer and recalled by a non-expert user (Brown and O'Leary, 1995). The characteristics of each filamentous organism can be programmed into the knowledge base of the expert system. The 15 parameters will serve as the key elements in the identification of the filaments. The searching scheme of the expert system can match the 15 keys with the correct filament electronically.

The expert system software will also assist the user through the microscopic observation of the wastewater sample and prompt the user to input the observations into the computer. During this process, the software will provide image instructions to the user, such as what a sheath looks like. The user of the computer just checks the yes or no option button on the screen, which corresponds to a certain characteristic observed under the microscope. After the microscopic observations are completed, the software searches the knowledge base and presents the typing result.

Objective of the Study

The objective of this research is to design and develop a personal computer based software to assist in identifying commonly observed filamentous bacteria in activated sludge wastewater treatment plants.

Procedure

The first step in this research was to develop the knowledge base for identifying filamentous bacteria found in wastewater treatment plants. The knowledge base contains the problem solving knowledge. The knowledge base was acquired from three main sources: *Manual on the Causes and Control of Activated Sludge Bulking and Foaming* (Jenkins et al., 1993), *Activated Sludge Microbiology* (Richard, 1989), and the author's several years of filaments typing experience on activated sludge. The knowledge base also includes different filamentous bacteria images which were developed by photographing images observed during microscopic examination of activated sludge samples.

The second step was to develop a searching scheme for the knowledge base. The searching scheme used in this project consisted of multiple filamentous bacteria searching trees. In Jenkins et al. (1993) manual, a dichotomous key for filamentous organism identification in activated sludge was provided. This dichotomous key was constructed utilizing the most commonly observed characteristics during typing of filamentous bacteria in activated sludge samples. Since some filamentous organism characteristics vary, the key does not address all possible variables (Jenkins et al., 1993). Sometimes these variable characteristics can lead to wrong identification. For example, *Thiothrix* can be identified in this key only when sulfur granules can be observed under the microscope. *Thiothrix* can appear without any sulfur granules in situ (Jenkins et al., 1993). Based on the writer's typing experience, a different type of searching key was developed. This new key addresses those variable characteristics necessary to lead to a

correct identification. If a characteristics is variable, an alternative identification path was constructed. Some characteristics, such as a sheath, are sometimes hard to observe under a microscope. Those hard to observe characteristics were not used in the searching trees.

The third step was the program development. The program implements the above developed searching scheme. The program design includes visual programming and code programming steps. Visual programming implements the program interface with the searching trees. Code programming steps implement the searching scheme of the knowledge base.

The last step was to evaluate the program. The goal was to evaluate the usefulness of the program. After the software was developed, an initial evaluation of the software was conducted by typing technicians, both experienced ones and amateurs. The purpose of the evaluation was to determine if the system really improved the technician's performance. One purpose of the project was to assist with filament typing by increasing the speed and accuracy of classification. The software was sent to one typing expert and five amateurs to exam the software. The expert evaluated the correctness of the new searching tree developed in this project and the usefulness of the program in typing filamentous bacteria in activated sludge samples. The information obtained from the amateurs evaluation of the program can assess if the program assisted with filament typing by increasing the speed and accuracy of classification.

The observed filament characteristics under a microscope are entered into the Filament Identification Worksheets. These work sheets are described in Table 1 and Table 2 of Chapter 3. These two tables consist of the observed morphological characteristics and staining results of filamentous bacteria under the microscope as well as sample identification information. These observed characteristics are used to identify the name of the filamentous organism. A computer program was developed to assist in the identification of observed filaments.

The computer program assists in typing of bacteria by implementing searching trees. The searching trees were developed based on the commonly observed filamentous descriptions provided in the literature (Jenkins et al., 1993; Richard, 1989). The program asks the user what has been observed under the microscope. The user inputs information observed under the microscope by clicks on the corresponding yes-no option buttons in the program. The program propagates the searching tree automatically based on the user's inputs.

The program was developed using Visual Basic Version 4.0 (Microsoft Corp., 1995), a PC-based software package that runs under the Windows® 3.1 operating system. The program provides a window graphic user interface. Images stored in the software and projected to the user, characterizing the filamentous bacteria, increase the confidence of user to make a yes-no selection on option buttons in the program. The quick

identification by the computer program makes typing of filamentous bacteria in activated sludge an easier task.

Summary of the Project

This project takes advantage of computer technology. A PC based software was developed to assist in typing filamentous bacteria in activated sludge samples. This software is window based and utilizes the mouse. On the screen, images of special filamentous bacteria characteristics and colorful text guide the user through the identification processes. The user of the software just points and clicks by using the mouse to input the microscopic observations into the program. Colored graphic interface and mouse clicks provide a faster and easier identification process than manually going through the Jenkins et al. (1993) identification flow chart and table.

Behind the screen is the program searching key. This searching key is different from the key used by Jenkins et al. (1993). This searching key addresses all the variable characteristics necessary to identify filamentous bacteria. All of these characteristics can be easily observed under 1000X phase contrast if they do exist. Some filamentous bacteria characteristics are variable and they are identified using different paths in the searching trees to insure they can be identified.

This searching key consists of four major searching trees. Each tree was constructed based on if attached growth and sulfur granules can be observed under 1000X phase contrast. Each tree consists of several sub-trees. This allows multi-point entry into the searching scheme. Therefore, variable filament characteristics can be handled easily.

The entire searching key was developed from a knowledge database and the writers typing experience. The knowledge database consisted of detailed descriptions of each filament type obtained from the literature. During the development of the searching trees using this database, two rules were followed. When querying the database to construct the searching trees, variable characteristics result in a filament being able to be identified using different paths. Second, if a characteristic can not be easily observed under 1000X phase contrast, based on the writer's experience, it was excluded from the searching trees.

CHAPTER II

REVIEW OF FILAMENTOUS BACTERIA IDENTIFICATION

Introduction to Activated Sludge Process

The activated sludge system is a biological technique used to remove organic matter from wastewater. The system consists of two processes. The first is the biological activity called the aeration process, and the second is called the settling process. The aeration process is the operation where biological treatment of the wastewater takes place. Inside the aeration basin a sizable population of microorganisms is maintained, for which the organics in the wastewater serve as the carbon and energy source. The microorganisms reduce the organics in the wastewater by converting the organic constituents to more microorganisms, H_2O, and CO_2. The purpose of aeration is to provide contact between the organics and the microorganisms in an oxygen rich environment suitable for microorganism growth. The purpose of the settling process is separation of the biomass generated by biological treatment from the liquid phase. The mixed liquor in the aeration process consists of large flocs which flow into the settling basin which is called a clarifier. Biomass settles to the bottom of the clarifier while the clear water that forms on the top of the clarifier flows out of the unit over the effluent weir.

Successful performance of the activated sludge process depends on the activated sludge mixed liquor formed in the system. The developed activated sludge mixed liquor is comprised of approximately 95% bacteria and 5% higher organisms such as protozoa, rotifers and higher invertebrates (Richard, 1989). An objective of the activated sludge process is to produce activated sludge floc from wastewater organics which will settle under gravity, in the clarifier, and leave a clear supernatant.

There are two levels of structure in activated sludge floc (Jenkins et al., 1993). They are termed the "microstructure" and the "macrostructure" (Sezgin et al., 1978). The microstructure floc contains only the "floc forming" bacteria (single-celled bacteria that form conglomerates). These flocs are usually small and settle rapidly, but can produce a turbid supernatant, called pinpoint floc. The macrostructure of activated sludge flocs contains filamentous microorganisms. Filaments form a backbone within the floc onto which the floc forming bacteria cling. This backbone provides the floc with strength and allows for a larger floc size. Figure 1 shows the microstructure and the macrostructure of an activated sludge floc.

Microstructure Macrostructure

Figure 1. Microstructure and Macrostructrue of an Activated Sludge Floc

However, excessive filamentous microorganisms in the activated sludge can lead to operational problems. Two of the most common problems are bulking and foaming.

Bulking and Foaming in Activated Sludge

A bulking sludge is defined as one which settles and compacts slowly (Pipes, 1979). The effect of bulking on plant operations is a high sludge volume index (SVI) (SVI > 150 ml/mg), very clear supernatant, low recycle solids (RAS) concentration, and in severe cases overflow of the sludge blanket (Jenkins et al., 1993). The cause of filamentous bulking is normally excessive filamentous organisms that extend from flocs into the bulk solution and interfere with the compaction and settling of activated sludge (Sezgin et al., 1978). Pipes (1967) stated that the main reason for poor settling properties of activated sludge is the presence of filamentous microorganisms, either free-floating in the mixed liquor or protruding from the flocs. Sezgin's study indicated that the quantity of filamentous organisms required to cause deterioration in settling properties is slightly greater than 10^7 μm/ml for activated sludge systems where a low DO filamentous organism such as *Sphaerotilus natans* or type 1701 was growing (Sezgin et al., 1978).

Foaming in the activated sludge process system can be caused by either non-degradable surfactants or by *Nocardia* spp. and sometimes by the presence of *Microthrix parvicella* filamentous microorganisms. *Nocardia* and *Microthrix* foams are persistent and hard to break mechanically. Foams carry large amounts of activated sludge solids to the surface of the aeration basin and sometimes it overflows the tank freeboard.

Investigations into the phenomenon of bulking and foaming have been an area of active research for many years. Research in this area is divided into identification of filamentous microorganisms in activated sludge and control of filamentous bulking and foaming. Research on filamentous microorganisms focuses on their identification and physiological properties. Jenkins (1992) reviewed the physiological properties of filamentous and floc-forming microorganisms found in activated sludge. Control of bulking and foaming has been investigated intensively by many researchers. The major control methods of filaments bulking and foaming have been detailed by Jenkins' manual (1993). The general approach to control filaments bulking and foaming can be summarized as identifying the causative filamentous organism(s), determining the probable cause(s) of the filamentous organism(s) growth based on the plant operating condition and wastewater characteristics, and making any operational changes to address the problem (Jenkins et al., 1993). This project focused on the identification of filamentous microorganisms.

Since the various filamentous organisms have a range of growth forms such as growing inside the floc, extending from the floc surface, and free in the bulk solution, the effect on settling properties of a given filamentous organism abundance will depend on the type of filamentous organism (Jenkins et al., 1993). Therefore, identification of various filamentous microorganisms becomes the first step in bulking and foaming control in the activated sludge process.

These filaments can appear under different operational conditions. Five specific causes of filament growth and bulking are currently recognized (Richard, 1989). Type

1701, *Sphearotilus natans (S. Natans), Haliscomenobacter hydrossis (H.hydrossis)* are typically caused by a low dissolved oxygen condition. *Microthrix parvicella (M. Parvicella), Nocardia spp., H. hydrossis*, Types 021N, 0041, 0675, 0092, 0581, 0961, and 0803 are typically caused by low organic loading rates. *Thiothrix* spp., Type 021N, 0041, and 0675 are typically associated with nutrient deficiency conditions. Septic wastes or sulfide conditions can cause *Thiotrix* spp., *Beggioatoa* spp., and Type 021N proliferation. Fungi will grow rapidly when the pH is below 6.0. Therefore, it is very important to know the identity of the filamentous microorganism so that an appropriate cure can be found.

The purpose of identification of filamentous organisms in activated sludge can be summarized as follows. The identification allows establishment that a settling or foaming problem is due to filament growth. From an identification of the most abundant filaments present in a bulking sludge, the cause for such growth can be estimated. Proper remedial actions can be initiated based on the cause of the filamentous bulking. Continuous microscopic observations can evaluate the effect of imposed operational changes on the types and abundance of filaments present to see if the appropriate remedial action has been taken.

Filamentous Organism Identification for Activated Sludge Processes

Eikelboom (1975) established the foundation for the identification of types of filamentous organisms in activated sludge and the rationality for resolving bulking problems. The typing method developed by Eikelboom did not use the standard

references given in traditional microbiology taxonomy manuals such as *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974) because many of the filamentous organisms found in activated sludge have not yet been assigned to a genus and species according to Bergey's Manual. Therefore, Eikelboom developed a relatively simple "identification" scheme specific for activated sludge filamentous organisms. This method relies on "in situ" observation of filament's morphology and staining reactions based on the observation of many activated sludge samples. Where a name did not exist for a filamentous organism, a number was assigned. Despite the fact that the types do not represent taxonomical species, their description meets all the practical needs for identification (Wanner and Grau, 1989).

David Jenkins further modified Eikelboom's filamentous organism typing method to develop what is considered to be the most common typing method in use today (Jenkins et al., 1993). The method is based on phase contrast microscopic observations of morphology and staining characteristics (Gram stain, Neisser stain and observation of sulphur granules). The filamentous microorganisms are classified into the so-called types according to the following features: cell shape, presence of sheath, filament length, morphology, presence or absence of sulphur granules, and staining characteristics. The taxonomic position of most of the types is uncertain (Wanner and Grau, 1989). The identification to types according to Eikelboom and Jenkins can be performed by trained non-microbiologists.

Scope of the Study

Over the past years research into bulking and foaming were mainly in the areas of identification of filamentous organisms, physiological study of filamentous bacteria, and control of filaments bulking and foaming. Typing of filamentous bacteria is the first step necessary to solve any bulking or foaming problem. It is very important to correctly identify filamentous bacteria in order to initiate an appropriate control strategies for bulking and foaming in the wastewater treatment plant.

The writer has been typing filamentous bacteria for four years. Jenkins (1993) and Eikelboom and van Buijsen (1981) have developed very useful filamentous organism keys to aid typing. However, based on the writer's experience with these keys, it was found that these keys were incomplete because they did not addressed all possible variables due to some filamentous organism's variable characteristics. Especially, for an inexperienced typing personal sometimes it is hard to identify a correct name for an filamentous organism by using Jenkins manual (Jenkins et al., 1993). Therefore, this project developed a new typing key and an easy to use PC-software which will guide the user through the typing processes.

CHAPTER III

MICROSCOPIC EXAMINATION OF ACTIVATED SLUDGE SAMPLES

Introduction

The software developed in this project displays various filamentous bacteria images on the screen. All these images show typical morphological and staining characteristics of filamentous bacteria observed in activated sludge. Images are stored in the software and projected to the user to increase the confidence of the user in making an identification.

To develop the images used in this project, microscopic examination of filamentous bacteria in activated sludge samples was conducted. Sludge samples were obtained from many different wastewater treatment plants (mostly industrial plants) across the United States. The features of the filamentous bacteria present in the activated sludge samples were observed under an Olympic microscope (Olympic BH-2). Photographs of the filamentous bacteria were taken using a camera (Minolta X-700) attached to the microscope.

Names for the filamentous bacteria in the activated sludge samples were identified using Jenkins' method (Jenkins et al., 1993). The following section details the microscope examination portion of this project.

Equipment and Supplies Used

An Olympic BH-2 microscope with 100X, 200X, 400X, 1000X phase contrast objectives was used. A 35mm Minolta X-700 camera with a built-in light meter was used to photograph microscopic images. Film used in the camera was 100 speed Kodak color film. During the typing of filamentous bacteria two staining techniques were used, the Gram stain and Neisser stain. A Gram staining set and the reagents necessary for the Neisser stain were made based on the recipes provided by Jenkins et al. (1993). Other supplies used in the experiment included plain microscopic slides and cover slips.

Microscopic Examination Procedure

The following microscopic examination procedures are based on Jenkins' manual (Jenkins et al., 1993). The microscopic examination for filamentous bacteria identification included the wet mount examination under phase contrast illumination at 100X and 1000X magnification, Gram stain examination under oil immersion at 1000X magnification with direct illumination (not phase contrast), and Neisser stain examination under oil immersion at 1000X magnification with direct illumination.

Wet Mount under Phase Contrast Illumination at 100X Magnification

Wet mount under phase contrast illumination at 100X magnification was used to examine if there were any filamentous bacteria in the sludge samples, the morphology of flocs, the abundance of filaments, and the effect of filaments on floc structure. The effect of filaments on floc structure can be classified as little or minimal effect, bridging, or open floc structure. The interfloc bridging is where filaments extended from the floc surface and hold the floc particles apart. In the case of open floc structure, the filaments grow mostly within the floc and the floc grows around and/or attached to the filaments. The floc becomes irregular or diffuse shaped and contains substantial internal voids (Richard, 1989).

The procedure for wet mount examination is described as follows:

1. Withdraw one drop of well mixed sample with a clean disposable pipette and place on a 25mm $\times$ 75 mm microscope slide.
2. Place a cover slip on the drop.
3. Observe the wet mount under phase contrast illumination at 100X magnification.

Record the observation on the work sheet provided in the following Table 1.

Table 1

Floc Characterization and Filamentous Organism Identification Work Sheet One

Plant Name:_____ Sample Location:_____

Sample Date:_____ Observation Date:_____

Filament Abundance: • None • Few • Some • Common

• Very Common • Abundant • Excessive

Filament Effect on Floc Structure: • Little or None • Bridging • Open Floc Structure

Morphology of Floc: • Round • Irregular • Compact • Diffuse

Wet Mount under Phase Contrast Illumination at 1000X Magnification

Wet mount under phase contrast illumination at 1000X magnification was used to determine which types of filamentous organisms are present in the sludge samples and their individual abundance. It should be noted that most of the time several types of filaments exist in the same sample. The procedure utilized for the wet mount under 1000X phase contrast is:

1. Under 100X phase contrast observation, select a spot with the filaments to be examined under 1000X phase contrast by scanning the microscope stage.

2. Turn away the phase ring and add one drop of oil onto the cover slip.

3. Change the microscope to the 1000X phase contrast setting by replacing the 100 phase ring.

4. Readjust the focus of the microscope.

5. Scan the sample with the mechanical stage control.

6. Characterize each filamentous organism present by looking at several filaments of each type and expressing the results as an "average."

7. Fill out the filamentous organism identification worksheet two provided in the Table 2.

Table 2

Floc Characterization and Filamentous Organism Identification Work Sheet Two

Plant Name:_____ Sample Location:_____

Sample Date:_____ Observation Date:_____

Filament No.	A	B	C	D
Branching				
Motility				
Filament Shape				
Filament Location				
Attached Growth				
Sheath				
Crosswalls				
Filament Diameter, µm				
Filament Length, µm				
Cell Shape				
Cell Size, µm				
Sulfur Deposits				
Gram Stain				
Neisser Stain				
Identification				

Gram and Neisser Stains

Gram and Neisser stains were used as two important identification tests in filamentous bacteria typing. Gram staining reagents and the reagents necessary for the Neisser stain can be made based on the recipes provided by Jenkins et al. (1993). The Gram and Neisser stain test procedures have been detailed by Jenkins et al. (1993). The general location and length of filamentous organisms in the wet mount, and the presence or absence of attached growth should be noted carefully so that the same filament types can be examined in the stained smears.

The Gram reaction was scored as strongly positive, weakly positive, variable, or negative. The Neisser staining procedure was scored as negative, positive, or negative with Neisser-positive granules. Most filamentous organisms observed in sludge samples are Gram and Neisser negative (Richard, 1989).

The filamentous organisms were classified on the basis of similarity in mainly morphological characteristics as described below in the activated sludge population as well as the Gram and Neisser stain results. The following features were noted and used to classify the organisms (Jenkins et al., 1993):

1. Presence or absence of branching; if present, whether it is true or false branching. True branching refers to cell branching where there is contiguous cytoplasm between trichomes. "A trichome (Gr. Trichos = hair) is a single, multicellular organism consisting of undifferentiated cells attached end-to-end like railway cars and clearly an entire multicellular structure" (Frobisher et al., 1974). In activated sludge the only

true branching filamentous bacteria are *Nocardia* spp. and *Nostocoida limicola*. The later is very rarely observed with a true branch. The only false branching filamentous organism in activated sludge is *Sphaerotilus natans*.

2. Gliding motility: present or absent. In activated sludge, *Beggiatoa* spp. and *Flexibacter* spp. are motile by gliding.

3. Filament shape: The filament shape is classified as straight, smoothly curved, bent, irregularly shaped "chain of cells," ie. a string of cells, coiled, or mycelial.

4. Location of filament can be extending from the floc surface, mostly within the floc, or free floating in the bulk solution between flocs.

5. Presence or absence of attached growth of epiphytic bacteria.

6. Present or absence of sheath? A true sheath is a clear structure exterior to the cell wall.

7. Cross wall (cell septa): present or absent.

8. Filament diameter measured in μm. It is important to note whether the average diameter is larger or smaller than 1 μm.

9. Cell shape can be square, rectangular, oval, barrel, discoid, round-ended rods (also called "sausage-shaped") or irregular.

10. Measured average size of cell (length and width) in μm.

11. Sulfur deposits: present or absent in situ or after applying the sulfur test. Sulfur granules appear as bright yellow "lights" under phase contrast illumination with most microscopes.

12. Gram and Neisser staining results.

CHAPTER IV

KNOWLEDGE BASE DEVELOPMENT OF FILAMENTOUS BACTERIA IDENTIFICATION PROGRAM

Introduction to the Knowledge Base Development

Knowledge base development is the foundation of this project. The knowledge base development includes knowledge acquisition and knowledge representation. Knowledge acquisition acquires the knowledge from human experts or other sources such as books and manuals. Knowledge representation encodes the knowledge for use by the expert system software. Common knowledge representation methods include rules, trees, and cases.

For this project, the knowledge base was developed from the author's several years of filamentous bacteria typing experience on activated sludge, Jenkins' *Manual on the Causes and Control of Activated Sludge Bulking and Foaming* (Jenkins et al., 1993), *Activated Sludge Microbiology* by Richard (Richard, 1989), and consulting an expert in typing filamentous bacteria, Chris K. Campana of Stover & Associates, Inc., who has a vast quantity of knowledge and experience in typing filamentous bacteria in activated sludge samples. The knowledge base contains the characteristics for identifying

commonly observed filamentous bacteria. The knowledge base was stored in a database developed in Microsoft Access®.

In the database, twenty database fields were created. The first field was named field of filamentous bacteria. The rest of the fields represented characteristics of the filamentous bacteria. The value of each field is a string of characters. The database had twenty-two records. Each record represented a filamentous bactrium. *Flexibacter spp.* was not included in the database because it is a small moving filament which is readily identifiable.

Many of the values of each field were derived from Jenkins' manual (1993) and Richard's manual (1989). Also, included was information based on the typing experiences of the writer and Chris K. Campana of Stover & Associates, Inc. For example, type 0041 often has a perpendicular attached growth on the trichome. This information was added by the writer. This special characteristic of attached growth on type 0041 was not mentioned by Jenkins manual (1993) and Richard (1989).

The following is a database view of filament type 0041. The left column is the field name for the database. The right column contains the value for the corresponding field. Other filaments viewed in the database can be found in the Appendix. If a field had two values, the first value means being most observed. A "variable" value means that field can be either positive or negative.

Filament Type: Type 0041

Gram Stain: positive or variable

Neisser Trichome: negative

Neisser Granules: negative or positive infrequently

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: none

Branching: no

Motility: no

Filament Shape: straight or smoothly curved

Filament Location: within the floc and extending from the floc

Filament Diameter: 1.4 to 1.6 μm

Filament Length: 100 to 500 μm

Attached Growth: yes (perpendicular), no

Sheath: yes and clear, tightly-fitting

Cell Shape: squares

Cell Size: 1.2 to 1.6 x 1.5 to 2.5 μm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: no

Note: Neisser stain positive occurs

The knowledge representation method selected for this project was the tree method. A network of nodes and relations is used to develop the trees. Each node contains a question about certain characteristics of filamentous bacteria in activated

sludge. The connection between each node defines the identification searching scheme. The searching trees were developed from the knowledge database. Details about the searching tree development are discussed in the next chapter.

Commonly Observed Filamentous Bacteria in Activated Sludge

The software was developed with the capability of identifying twenty three commonly observed filamentous bacteria in activated sludge processes. They are listed in Table 3. A detailed characteristic description of each filament is presented in the Appendix. The Appendix includes twenty two filamentous descriptions. The description of *Flexibacter spp.* is not included in the Appendix since it is a easy identifying filament which is a small moving filament. The infrequently observed filament types 1702, 1852, and 0211 are not identified by the software. Fungi, *Cyanophyceae*, and *Bacillus* spp. are very easy to identify and are not included in Table 3.

Table 3

Filamentous Bacteria Identified in the Program

Filament Name
Sphaerotilus natans
Type 1701
Type 0041
Type 0675
Type 021N
Thiothrix I, II
Type 0914
Beggiatoa spp.
Type 1851
Type 0803
Type 0092
Type 0961
Microthrix parvicella
Nocardia spp
Nostocoida limicola I, II, III
Haliscomenobacter hydrossis
Type 0581
Type 1863
Type 0411
Flexibacter spp.

CHAPTER V

SEARCHING TREE DEVELOPMENT

Introduction

Identification of unknown filamentous bacteria in activated sludge is typically accomplished by means of a dichotomous taxonomic key. Such a key presents the user with progressive choices between pairs of alternative characteristics. The user examines the unknown filamentous bacteria, then chooses one alternative or the other from the dichotomous taxonomic key. Selection of the most applicable alternate leads to other pairs of alternatives and ultimately to a specific filamentous bacteria name.

A dichotomous key from the *Manual on the Causes and Control of Activated Sludge Bulking and Foaming* (Jenkins et al., 1993) is shown in Figure 2. This key tree is a modification of the filamentous organism identification key in *Bulking of Activated Sludge: Preventative and Remedial Methods* (Chambers and Tomlinson, 1982) given by Eikelboom and Van Buijsen, with changes made to emphasize the need for the observation of cell crosswalls, which can depend on the quality and adjustment of the microscope used; and to include some filamentous organisms in the key twice where an important characteristic is variable. Figure 3 shows the identification key by Eikelboom and Van Buijsen (1981).

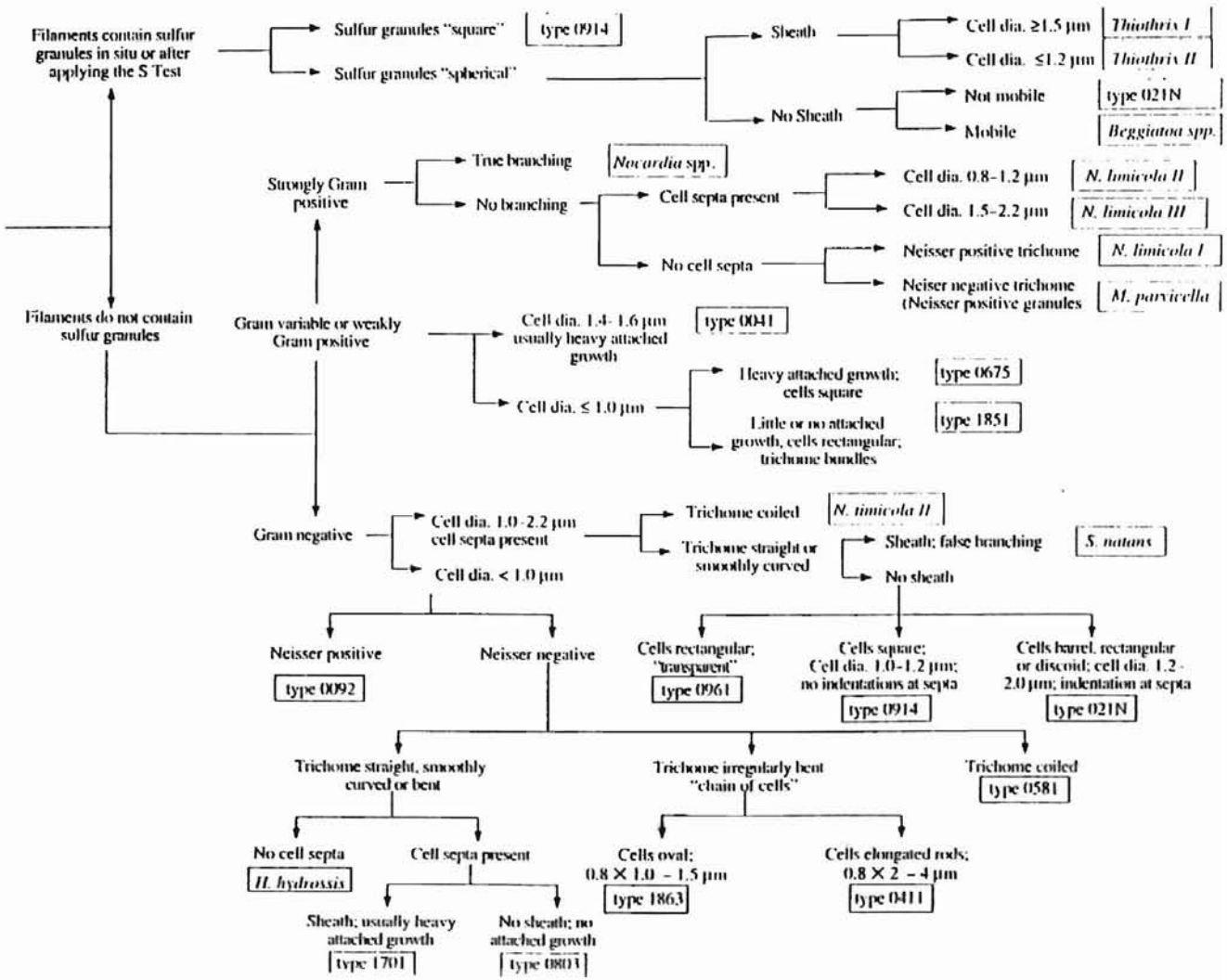

Figure 2. Searching Key Developed by Jenkins et at (1993)

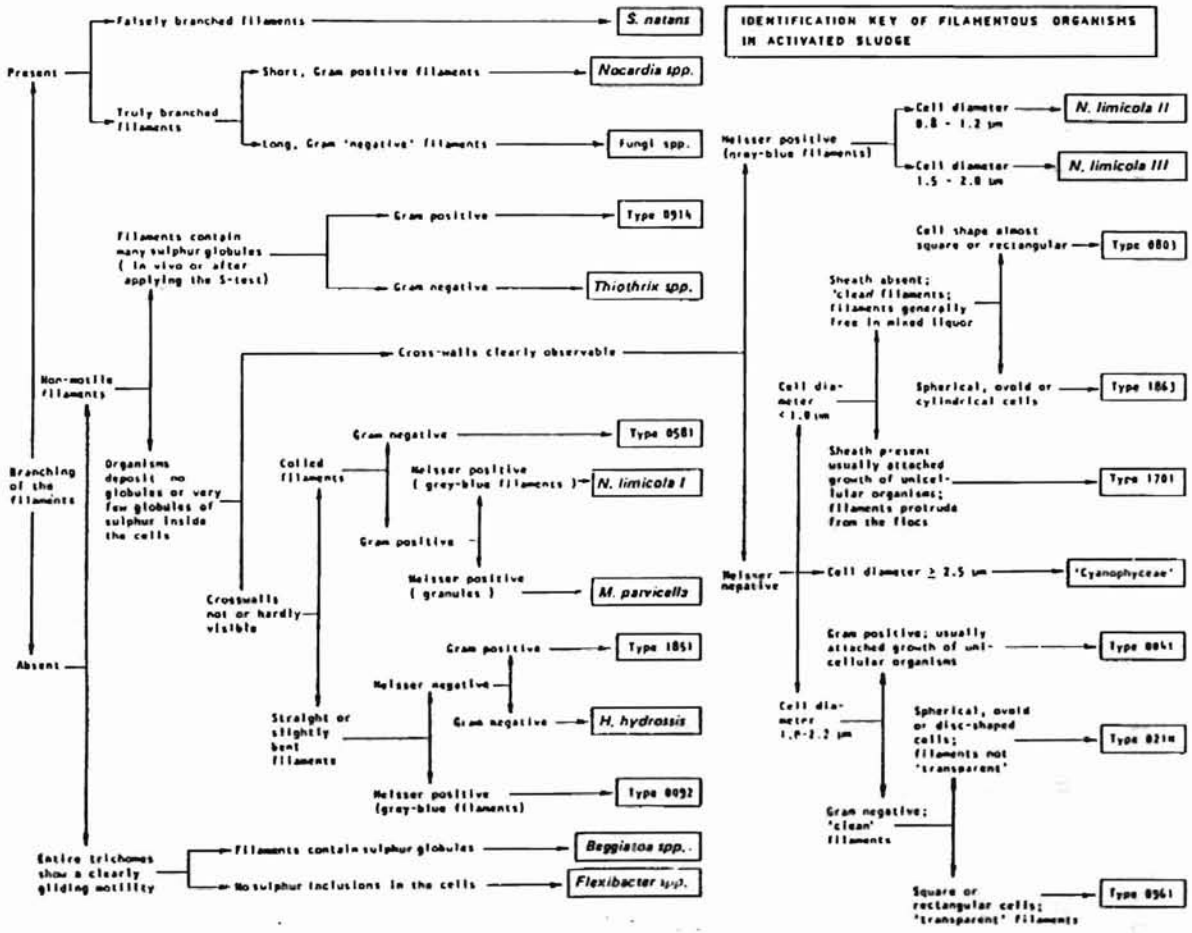

Figure 3. Searching Key Developed by Eikelboom and van Buijsen (1981)

The use of these keys (Figures 2 and 3) can be incomplete or inappropriate sometimes because some filamentous organism characteristics vary and the key cannot always address all possible variables. The filamentous bacteria determined by these keys have to be checked carefully with the photographs and short descriptions of the filaments provided in the manual. These short descriptions about each filamentous bacteria provide better guidance than do the keys. Sometimes the filamentous bacteria identified using the keys does not match the short descriptions and photographs provided by the manual. An example of this scenario is given in Test 2 of Chapter VII. In Table 7 of Chapter VII, *Thiothrix* was identified as 0961 using Jenkins' key, which does not match the short description by the manual. Therefore, the observed filamentous organism is difficult to assign a name, type, or genus designated in the key. In practice, successful identification of filamentous bacteria relies on learning to recognize special features that provide clues to the filamentous organism type.

Searching Tree Development

The central part of this project was to develop a set of searching keys based on some special features which are unique to specific filamentous organisms. These special features, such as attached growth and sulphur deposits on filaments' trichome, should be unique to an individual filament or a group of filaments. Based on literature (Jenkins et al., 1993), it is not hard to find special features which are morphological characteristics of filamentous bacteria. Also, these special features should be always observable under 1000X phase contrast.

In this project, a different set of filamentous identification keys were developed based on the special features unique to each filamentous bacteria or to a group of filamentous bacteria. The special features include attached epiphytic growth, sulfur granules in situ, transparency, branching, motility, rigidly straight, chain of cells, trichome bundles, sausage shaped cells, and square sulfur granules in situ. These special features were derived from querying the database built during the knowledge development phase of this project.

The filamentous identification key based on these special filamentous bacteria features can be used to give a quick clue in the identification process. By implementing this identification key into an interactive window program, the user can propagate through the identification key trees once and check the result with the short description provided in Jenkins et al. manual (1993). If the filament name identified did not match the short description in the manual, the user can go back to the program and check the special features selected in the program under the microscope.

The filamentous identification key developed in this project consists of four independent trees. In the first case, a tree was developed based on the condition of neither attached growth nor sulfur granules observed associated with the filamentous bacteria in the activated sludge sample. The second searching tree was developed for filamentous bacteria with attached growth. The third searching tree was for filamentous bacteria with observed sulfur granules in situ. For the filamentous organism observed with attached growth and sulfur granules in situ, there is only one bacterium (Type 0914). This is the fourth searching tree which contains only one node.

After carefully querying the knowledge base developed in the last chapter, to their filamentous bacteria can be identified using the four searching trees. In the searching tree based on no attached growth and no sulfur granules, twenty three commonly observed filamentous organisms are included since some filamentous bacteria can exist with either the presence or absence of both attached growth and sulfur granules in situ. In the the searching tree associated with attached growth, only seven filamentous bacteria are included. They are *S. natans*, Type 1701, Type 0041, Type 0675, *H. Hydrossis*, Type 0914, and Type 1851. In the third searching tree, for sulfur granules in situ, five possible filamentous bacteria, Type 021N, *Thiothrix* I, *Thiothrix* II, Type 0914, and *Beggiatoa* are included. In the case of both attached growth and sulfur granules in situ observed associated with filamentous bacteria, there is only one filament called Type 0914.

Each searching tree then was developed by further querying the knowledge base using the characteristics of filamentous bacteria. The results of this further query of the knowledge base are presented in Figure 4 to Figure 8. Figure 4 to Figure 6 depict the resulting trees with no attached growth and no sulfur granules in situ. Figure 7 shows the searching tree for filaments with attached growth observed. Figure 8 illustrates the searching tree for filaments with sulfur granules in situ. The searching tree for filament with both attached growth and sulfur granules in situ observed is not presented in a figure because only Type 0914 is in this fourth tree.

Figure 4 started with six morphological characteristics of filamentous bacteria. These characteristics are transparency, branching, motility, rigidly straight filaments, oval cells, and trichome bundles. All of these characteristics can be easily observed under

1000X phase contrast magnification, if they exist. These characteristics each lead to their respective sub-trees. This construction of the tree allows multipoint entries into the whole searching tree. Inside the branching characteristic sub-tree, three filaments are included. They are *Nocardia, Limicola* II, and *S. natans*. *S. natans* is the only filament associated with false branching. *Norcardia* and *Limicola* II can be identified by either Neisser staining or filament length. In Figure 4, the dotted lines around the Neisser positive characteristics indicate a possible searching path to identify *Limicola* II. *Limicola* II appears Neisser positive most of the time (Jenkins et al., 1993). However the correct answer can be derived from the length characteristics. Inside the sub-tree of oval cells, two filaments, Type 1683 and Type 0411, are identified using two different filament lengths, which can be easily measured under the microscope. Trichome bundles sub-tree indicate that Type 1851 is observed more commonly in bundles of intertwined filaments.

Figure 5 is a continuation of Figure 4. It starts with Neisser positive stain results. In the sub-tree of Neisser positive, six filaments are included. Type 0041 and 0675 may have Neisser positive trichome covering (N+ covering) when present in industrial wastewater activated sludge systems (Jenkins et al., 1993). Type 0041 and 0675 can also be identified when the Neisser stain is negative. This is one of the differences between this searching tree and the trees developed by Jenkins et al. (1993). *Limicola* II may be identified in Figure 5 using two different paths depending on what is observed under the microscope.

Figure 6 identifies six filaments. Figure 6 is a continuation of the identification process of Figure 5. In this figure, *Thiothrix* can be identified. This is another

improvement over previous searching trees. From Jenkins et al. manual (1993), *Thiothrix* I and II can sometimes appear with no sulfur granules. This tree addresses this unique identification path for *Thiothrix* I and II. *Limicola* II can be identified again using this part of the tree.

Figure 7 is a unique searching tree adapted from the ones previously developed by both the Eikelboom and Jenkins groups. This tree was developed based on the presence of attached growth. From the author's typing experience, many times activated sludge samples contain filaments with attached growth. There are only seven filaments associated with attached growth (Jenkins et al., 1993). An independent tree started from attached growth can increase the speed and accuracy of filament identification.

Figure 8 contains all the possible filaments associated with sulfur granules. This tree is a modification of the corresponding part of Jenkins' identification key (Jenkins et al., 1993). In this tree, Type 021N and *Thiothrix* can be identified twice using different searching paths. This tree is so constructed because the characteristics of sheaths are sometimes hard to observe under the microscope. Type 021N is not sheathed. However, when cells are lysed, the heavy cell wall of 021N can remain and be confused as a sheath. The searching tree handles this observation error by including the 021N into the sheath sub-tree first. Then, Type 021N is further identified by cell shape.

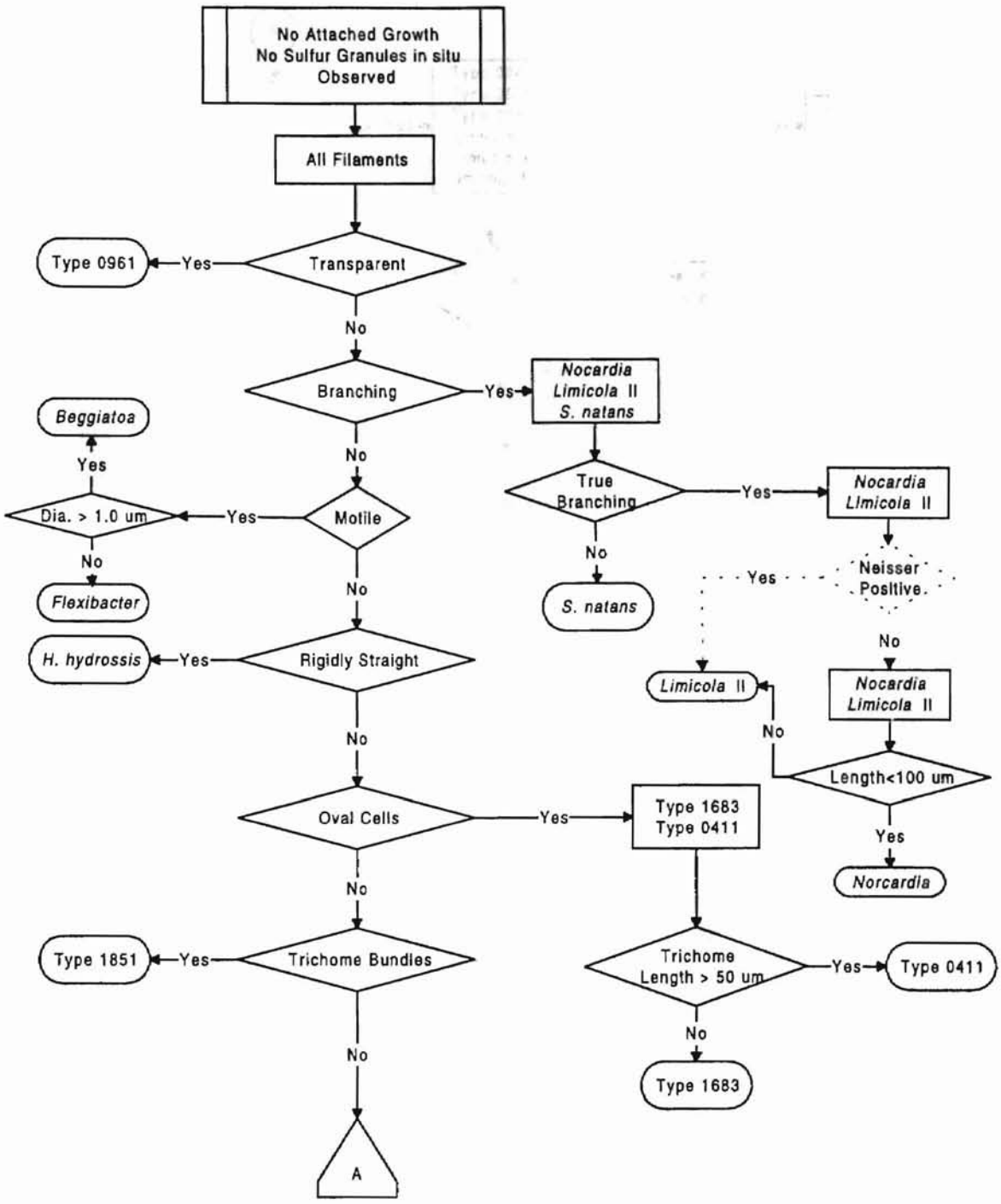

Figure 4. Searching Tree Part 1 for All Commonly Observed Filamentous Bacteria

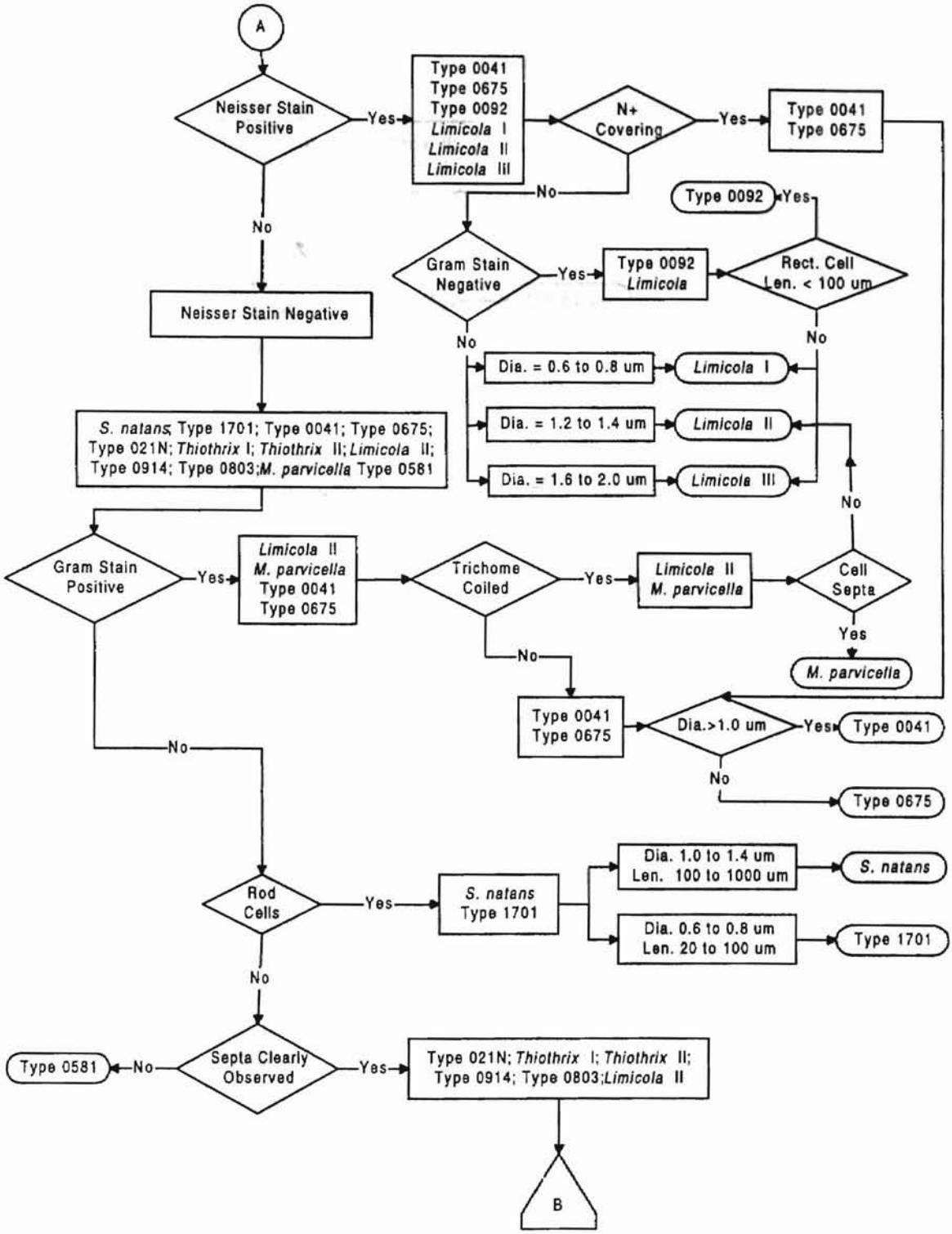

Figure 5. Searching Tree Part 2 for All Commonly Observed Filamentous Bacteria

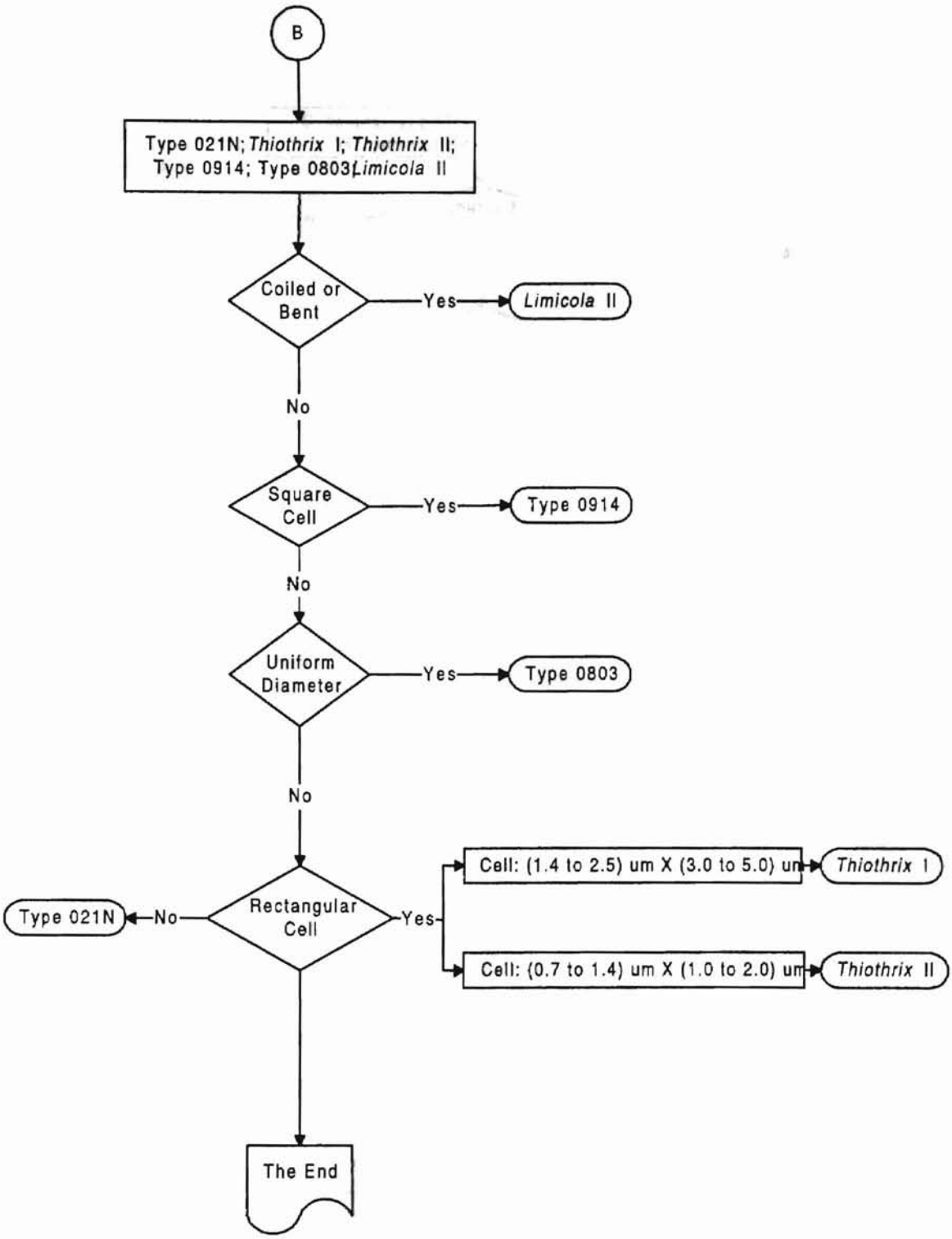

Figure 6. Searching Tree Part 3 for All Commonly Observed Filamentous Bacteria

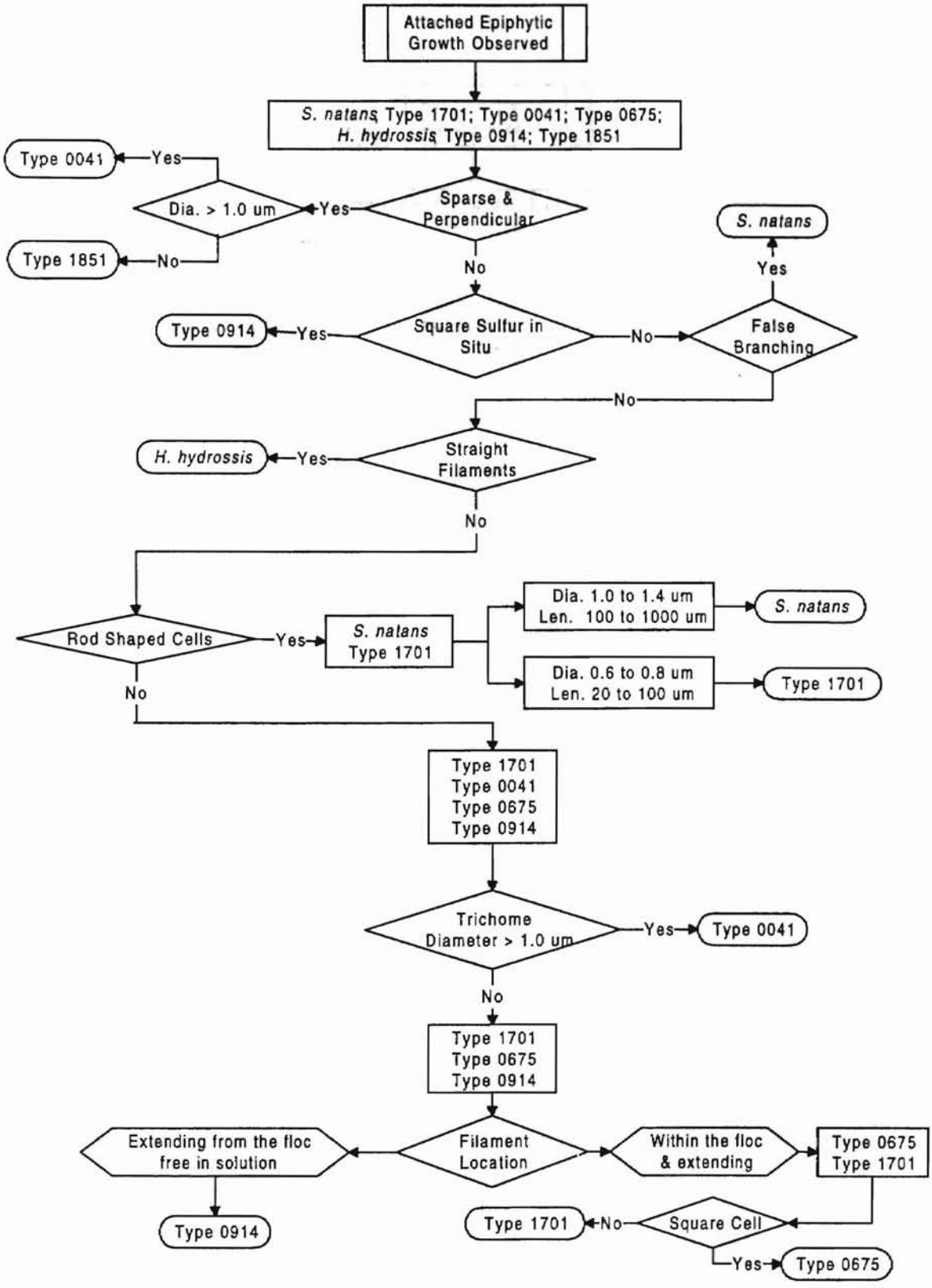

Figure 7. Searching Tree for Filamentous Bacteria with Attached Growth Observed

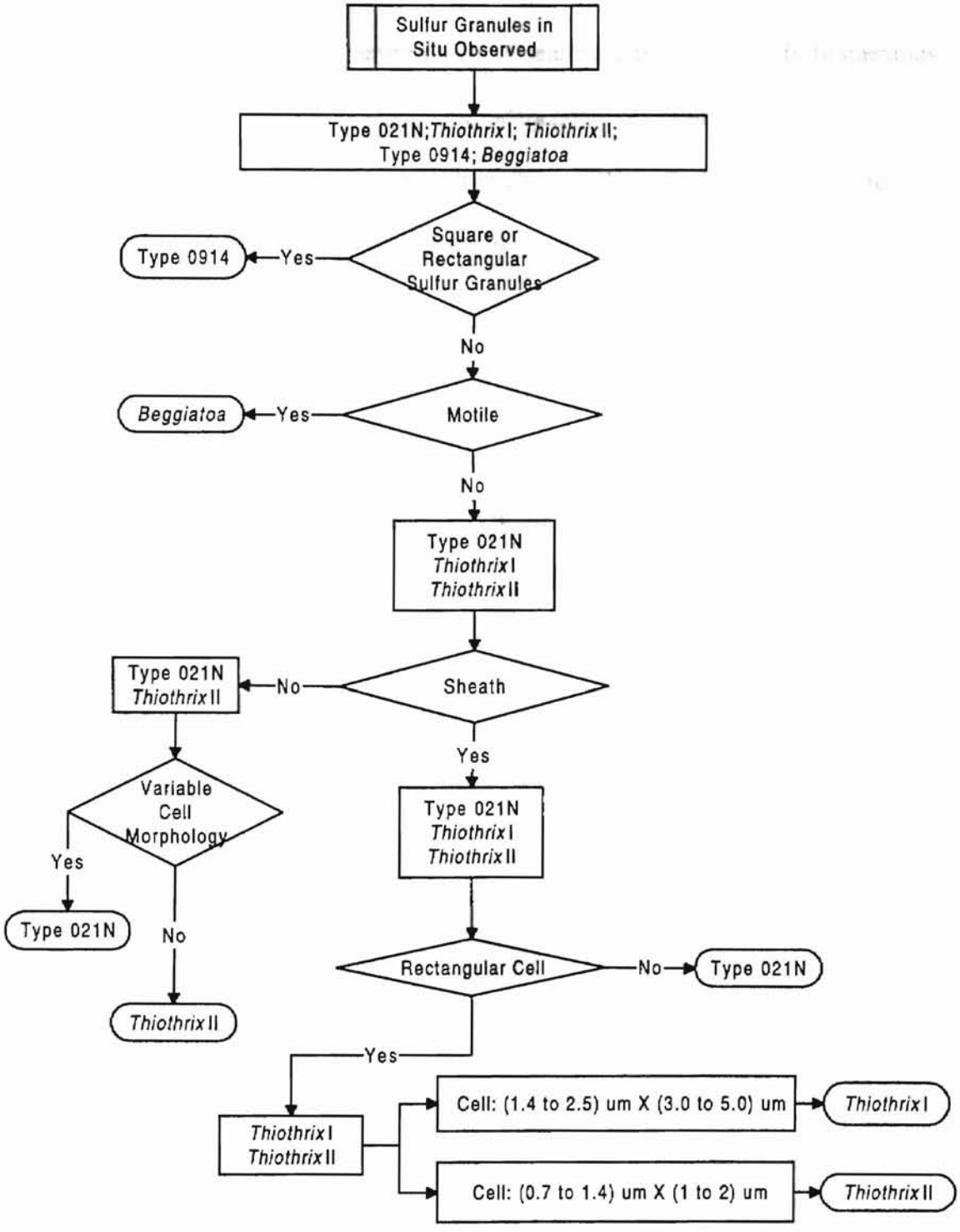

Figure 8. Searching Tree for Filamentous Bacteria with Sulfur Granules in Situ

Summary of the Searching Trees Developed

This chapter discusses the development of searching trees to identify filamentous bacteria in wastewater treatment plants. Four independent searching trees were developed based on the knowledge base built in Chapter IV. All of these trees were developed based on some special features unique to each filamentous bacteria or to a group of filamentous bacteria. Two key features, attached growth and sulfur granules, divided the identification process into four independent searching trees.

The trees developed in this project differ from the keys developed by Jenkins et al. (1993) and Eikelboom and van Buijsen (1981) in the following ways. Four searching trees allow multiple entry points into the identification processes. If attached growth appears, only seven filamentous bacteria need to be considered. Since attached growth is very easy to observe, a separate tree for attached growth is very useful in identification. For example, filament 0041 is a very common filament. Most of the time, it has sparse and perpendicular attached growth. In Figure 7, filament 0041 can be identified very quickly if perpendicular attached growth is present. Using Jenkins' key, it can only be identified after completion of a Gram stain. Since the Gram stain characteristics of type 0041 are variable, it is best to rely on the morphological features for identifying these filaments (Jenkins et al., 1993). All the filaments included in the attached growth searching tree were further classified based on special morphological characteristics, which are perpendicular attached growth, square sulfur granules, false branching, straight fine filaments, and rod shaped cells. If any of the above special characteristics exists, identifying a given filament only needs two to three steps.

If in situ sulfur granules are observed, the identification process goes into a searching tree for filamentous bacteria with sulfur granules in situ, which is presented in Figure 8. This tree contains the same filaments as the Jenkins key does. However, in Jenkins' key, presence or absence of a sheath is used as a key characteristic in identification. Sometimes, the sheath is a characteristic difficult to observe under the microscope. Therefore, Figure 8 identifies *Beggiatoa* by its motility feature and differentiates the 021N from *Thiothrix* by cell shape if a sheath can not be observed.

If attached growth or sulfur granules are present on the filaments, then Figures 4 to 6 are used. This searching tree is the biggest tree among the four searching trees. It identifies all twenty three filaments. This tree is different from keys developed by previous researchers in the following ways. Figure 4 utilizes some special features unique to an individual filament or a group of filaments to identify the filamentous bacteria. If any of the features presented in Figure 4 can be observed under the microscope, the identification process only takes two to three steps. However, when using Jenkins' key, one must go through the complete searching tree for all of the filaments identified in Figure 4. From the author's typing experience, filaments identified in Figure 4 are fairly easy to identify. They can be recognized when first seen under the microscope. Also, *Beggiatoa* can not be identified when sulfur granules are not present on the trichome in Jenkins' key.

If none of the above special features presented in Figure 4 exists, Figure 5 is used to further identify filaments. Figure 5 utilizes Gram positive, Neisser stain positive, and rod shaped cell characteristics to identify filaments. Based on Jenkins' manual, six

filaments can stain Neisser positive and four Gram positive. They are identified using the Neisser stain positive sub-tree and Gram stain positive sub-tree, respectively. Two filaments, *S. natans* and type 1701, are rod shaped cells. They can be readily identified by their different size.

Figure 6 is a continuation of Figures 4 and 5. It is used to identify filaments when they appear to be Neisser stain negative, Gram stain negative, have cell septa clearly observed, and no attached growth and sulfur granules. The filaments identified in this figure are type 021N, 0914, 0803, *Limicola II*, and *Thiothrix*. Figure 6 allows identification of *Thiothrix* when sulfur granules are not present in situ. In Jenkins' key, *Thiothrix* can not be identified if *Thiothrix* appears to contain no sulfur granules in situ.

CHAPTER VI

PROGRAM DESIGN

Program Objectives

The main objective of this program was to implement a filamentous identification scheme to assist in the typing of commonly observed filamentous organisms in the activated sludge process of a wastewater treatment plant. Various program designs can be employed to accomplish this goal. To ensure a quality program several goals were identified before the design process began. These goals considered how potential users would accept the program.

The first goal was to eliminate the keystrokes when using the computer. Programs that require large numbers of keystrokes add greatly to the time it takes to identify a filamentous organism. The user was assumed to know how to use a mouse under the windows environment.

The second goal was to make the decision process as easy as possible. This was accomplished by adding explanatory images to the technical terms whenever possible (for example, displaying an image that shows what filaments with or without sulfur granules in situ look like).

The third goal was to make the program attractive and appealing to the eye and easy to read. This was accomplished by implementing the program under the windows environment and adding filamentous bacteria images on the screen. Questions to be answered always appear in blue color. When a question is answered, the program automatically prompts the next question by highlighting its title with blue color.

The fourth requirement was that the program implement the filamentous identification accurately. To guarantee the correct implemention of the searching tree, each selection the user makes remains on the screen during the most current identification session. In order to visualize the searching tree, each screen shows as many option buttons as possible during an identification session.

Because most wastewater treatment labs do not have a sophisticated computer, the fifth and last goal required the program to run on a personal computer with Microsoft Windows 3.1.

Development Software Selection

To meet the above design goals, the developed software uses Microsoft Visual Basic 4.0 16-bits version. This software was developed to provide results in an easy to interpret manner and was structured to be interactive and user friendly in the Microsoft Windows 3.1 environment. All of these features help to make this program easy to learn for those with little computer training.

The visual programming step implements the portrayal of the filamentous bacteria identification searching scheme on the screen. According to the searching key developed in chapter five, the key is divided into four trees. In Figure 9, the four trees are shown as four option buttons with four explanatory images.

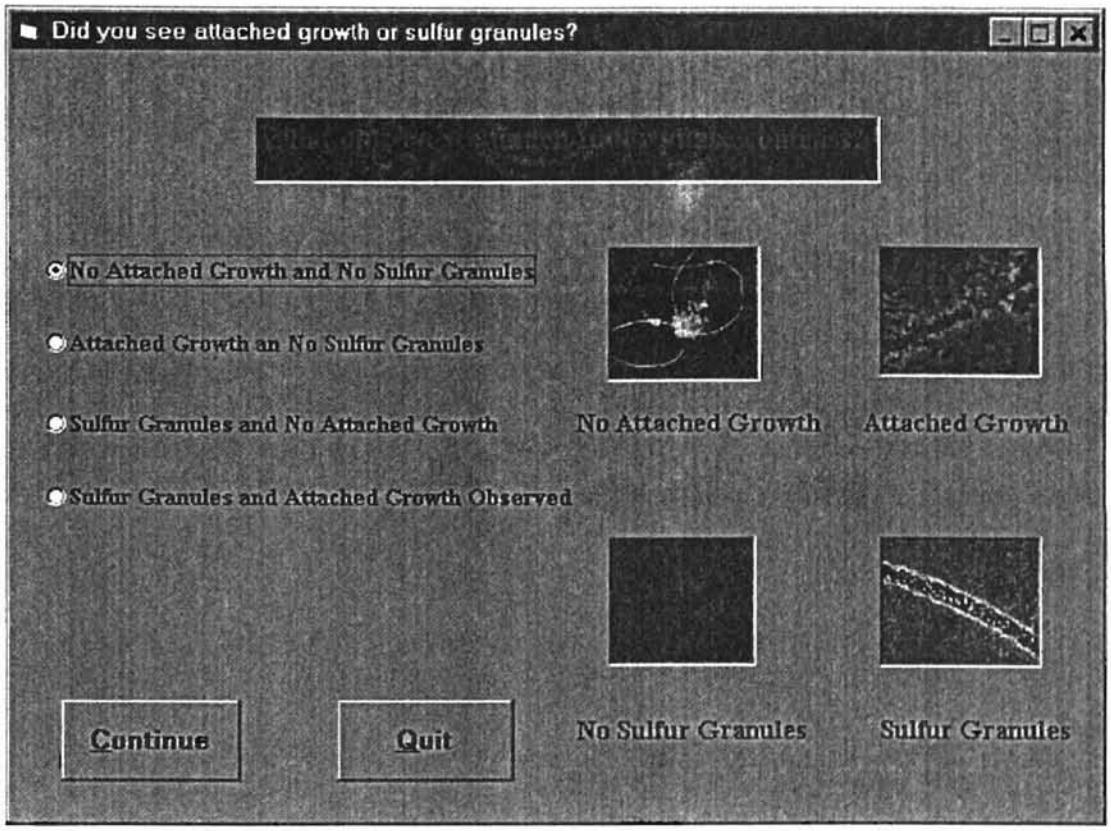

Figure 9. Attached Growth or Sulfur Granules Screen

In Figure 9, the user can choose any one of four option buttons just by clicking on the icon. The four images on the screen show what attached growth and sulfur granules associated with filamentous bacteria look like. These images help the user to make the correct choice by comparing the images on the screen with microscope observations.

Choosing one of the different option buttons on this screen allows the user to go into one of the four different searching trees developed in Chapter five.

Figure 10 shows the screen implementation of the portion of the identification searching tree given in Figure 4. If the branching option button is selected, the screen can further change into Figure 11, Figure 12, or Figure 13, respectively. If the oval cells option button is selected, the screen changes into Figure 14. In this case filaments type 0411 and type 1683 can be identified. All of these screens together implement the searching tree illustrated in Figure 4.

Figure 15 and Figure 16 implement the second page of the first searching tree. Figure 17 shows the third page of the first searching tree. Figure 18 shows the screen design for the attached growth searching tree. Figure 19 displays the screen design for the sulfur deposit filamentous bacteria identification tree. Figure 20 shows a test run of Figure 19.

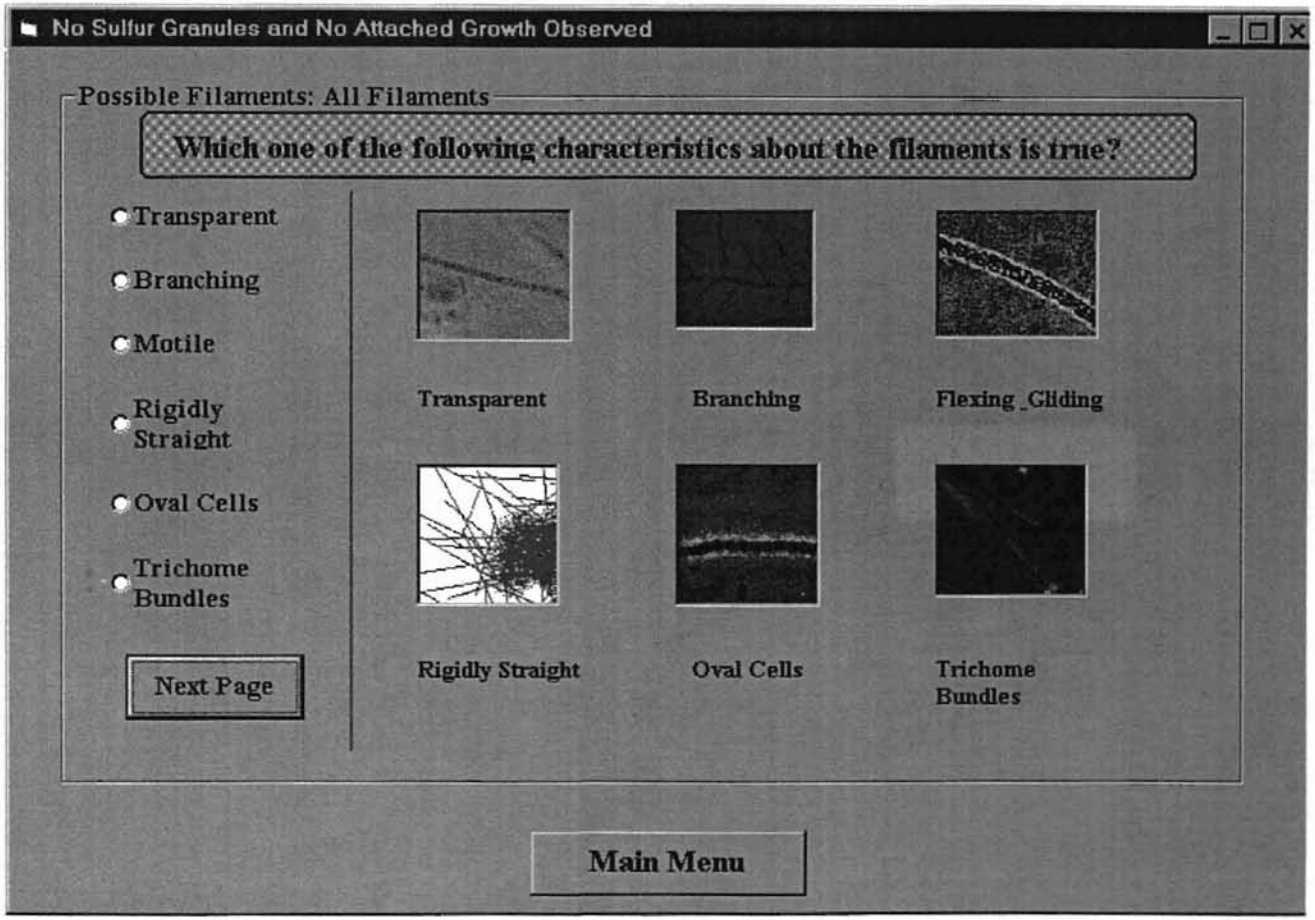

Figure 10. First Searching Tree (Page 1)

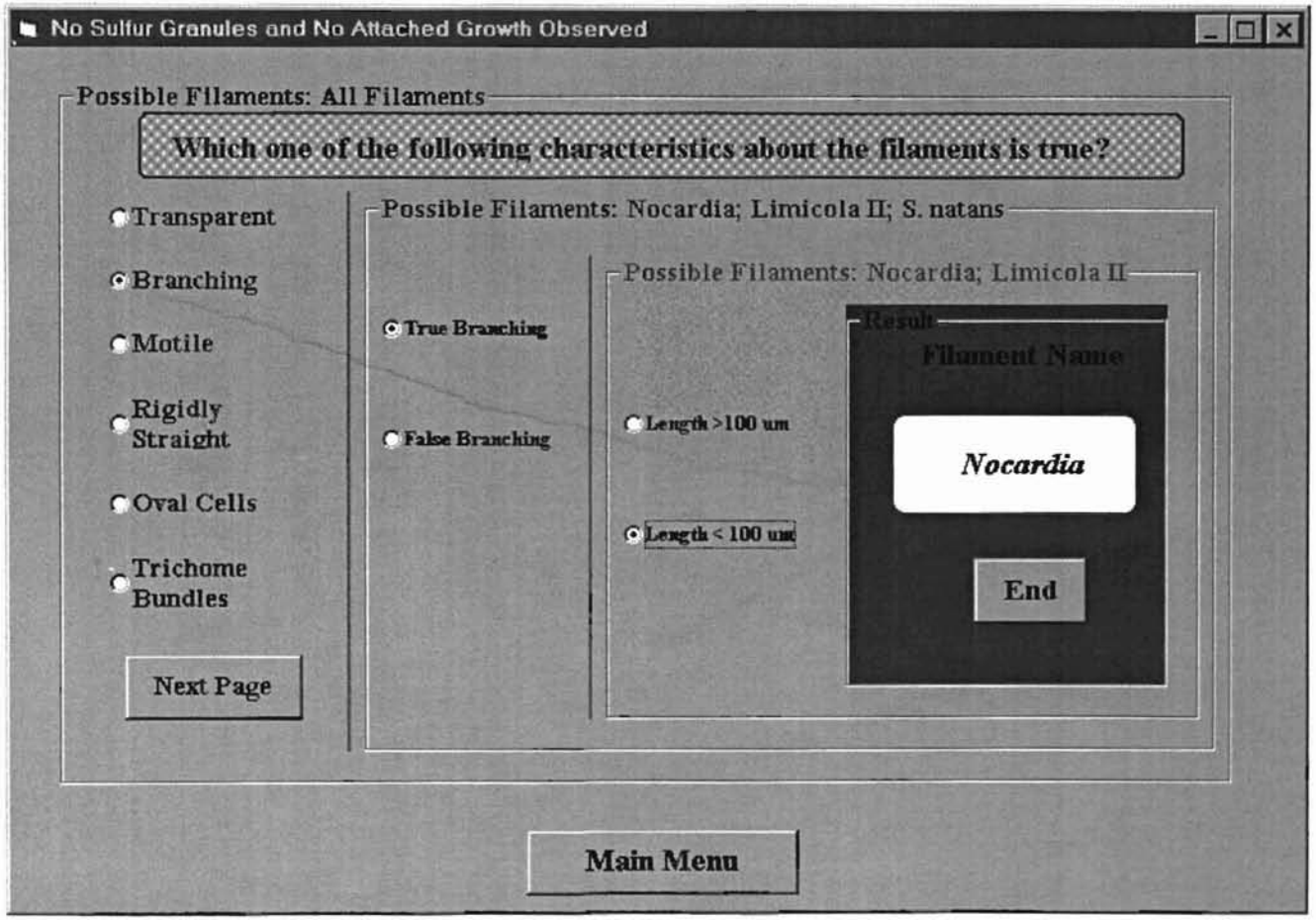

Figure 11. First Searching Tree (Page 2)

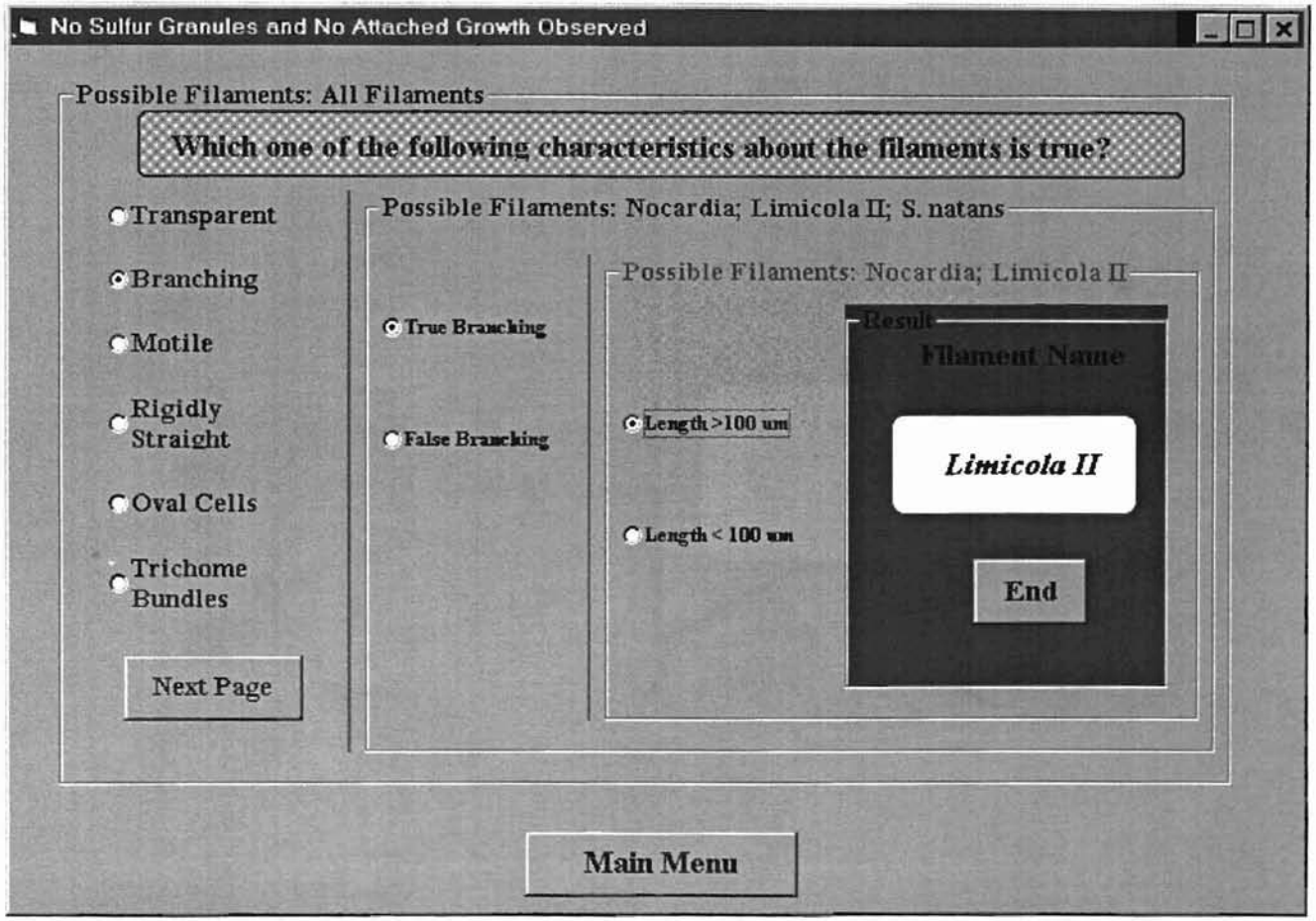

Figure 12. First Searching Tree (Page 3)

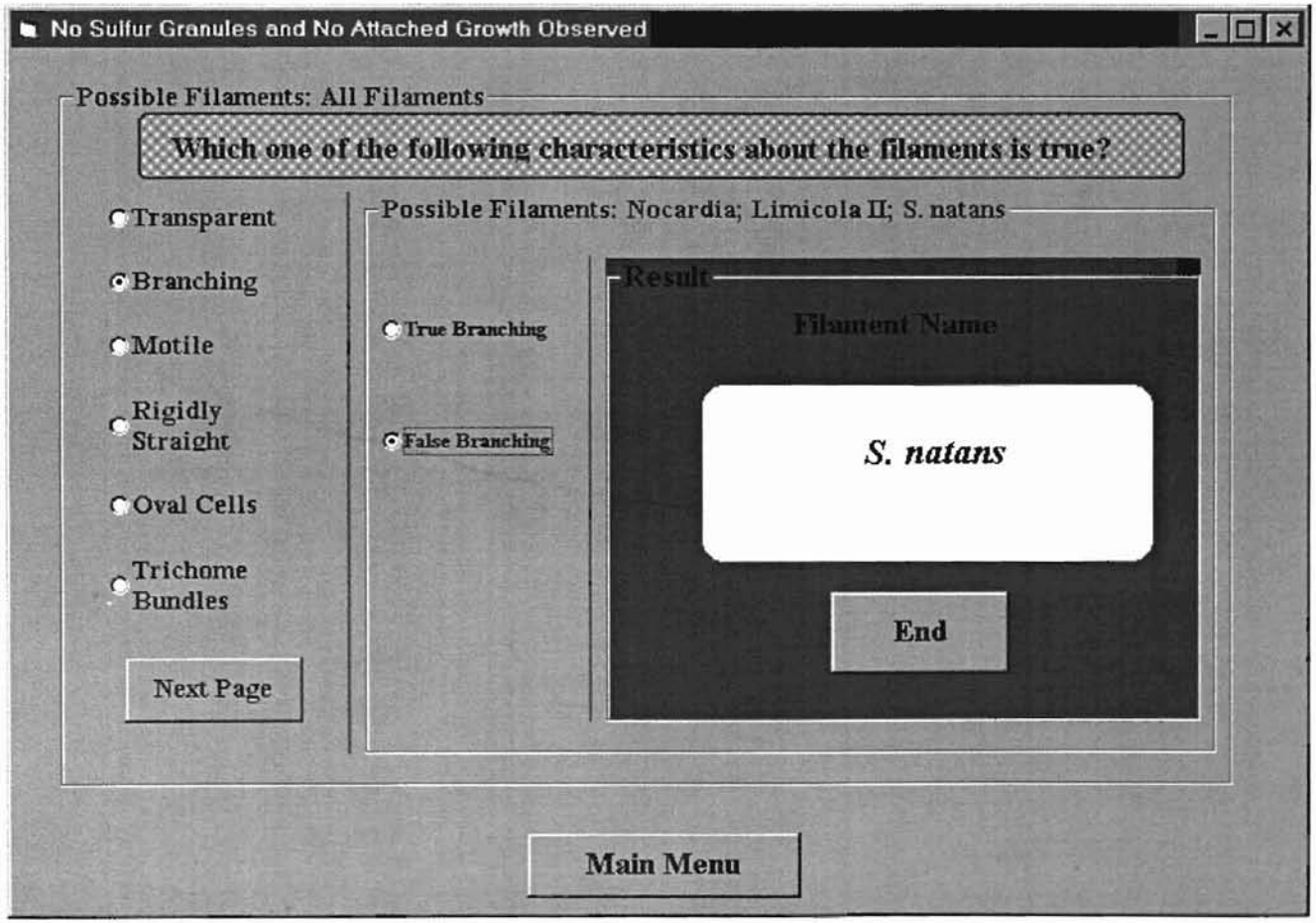

Figure 13. First Searching Tree (Page 4)

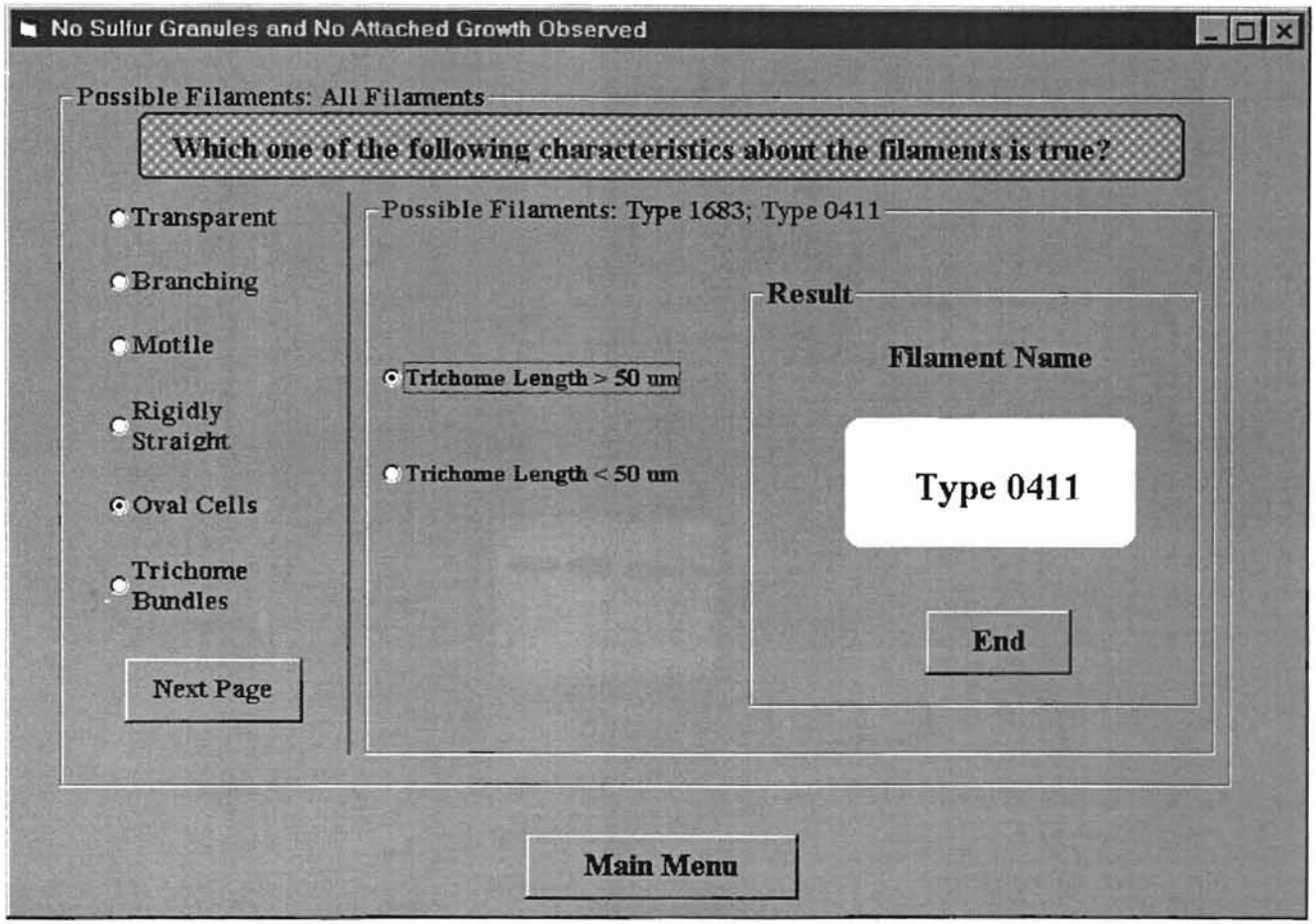

Figure 14. First Searching Tree (Page 5)

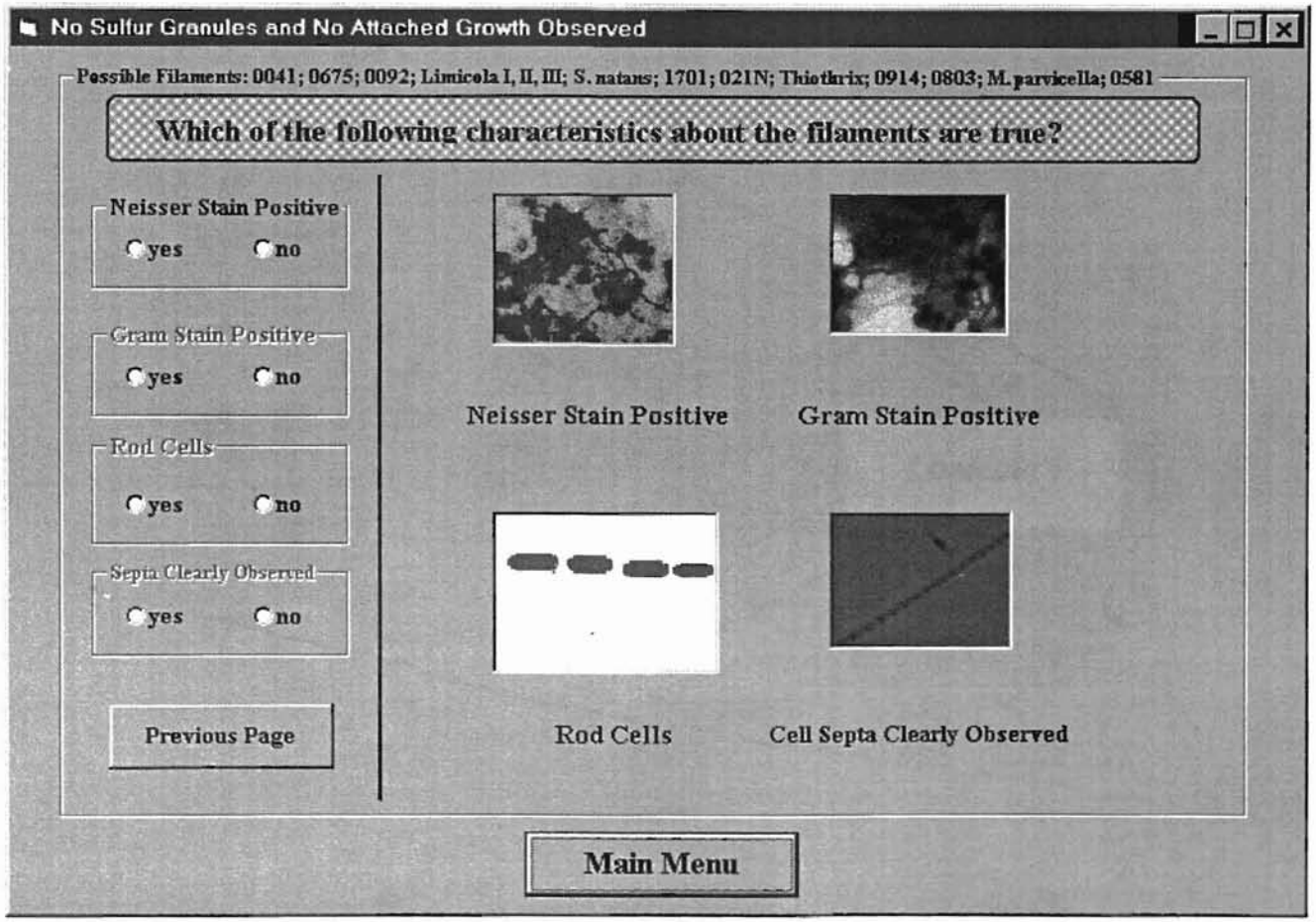

Figure 15. First Searching Tree (Page 6)

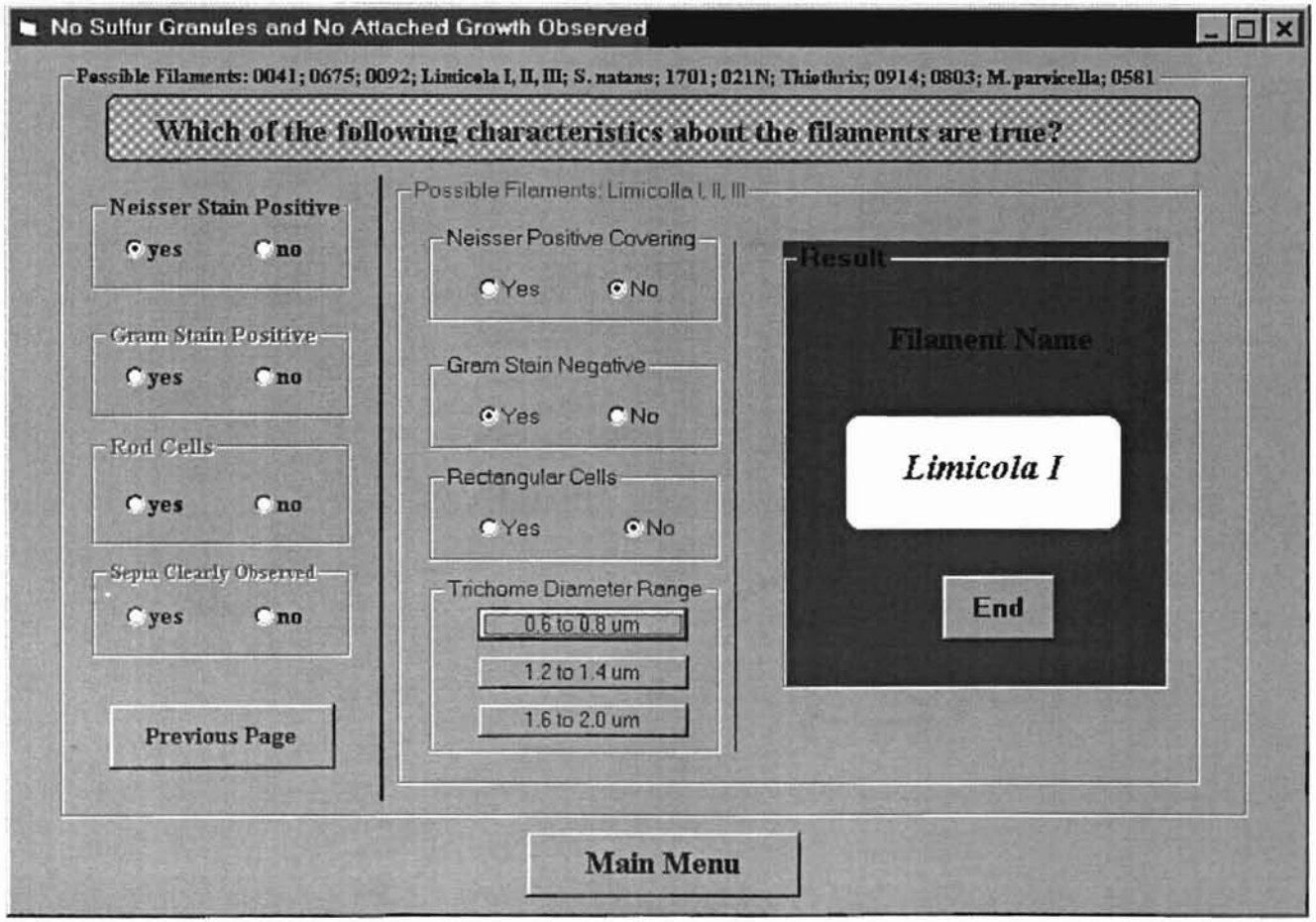

Figure 16. First Searching Tree (Page 7)

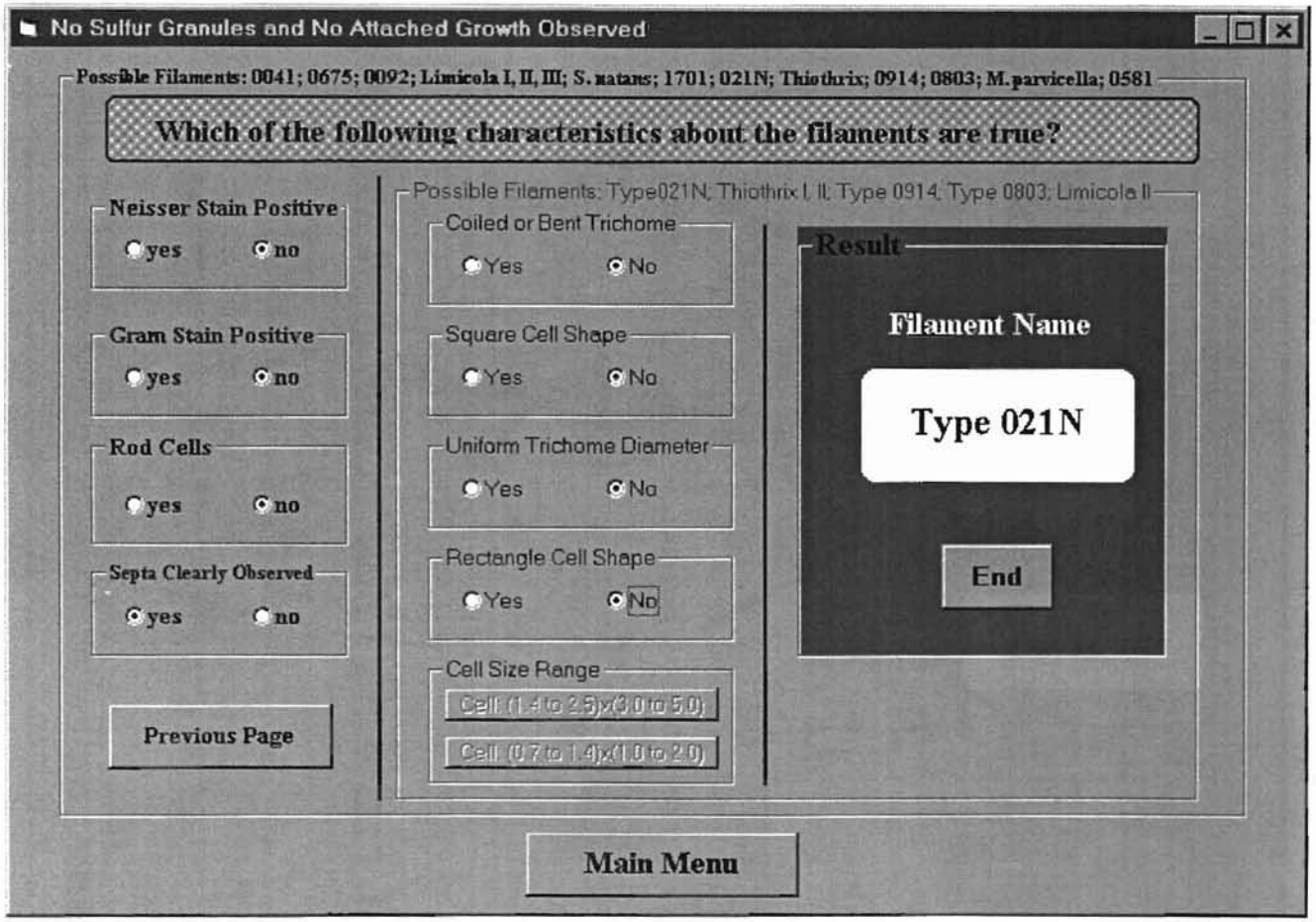

Figure 17. First Searching Tree (Page 8)

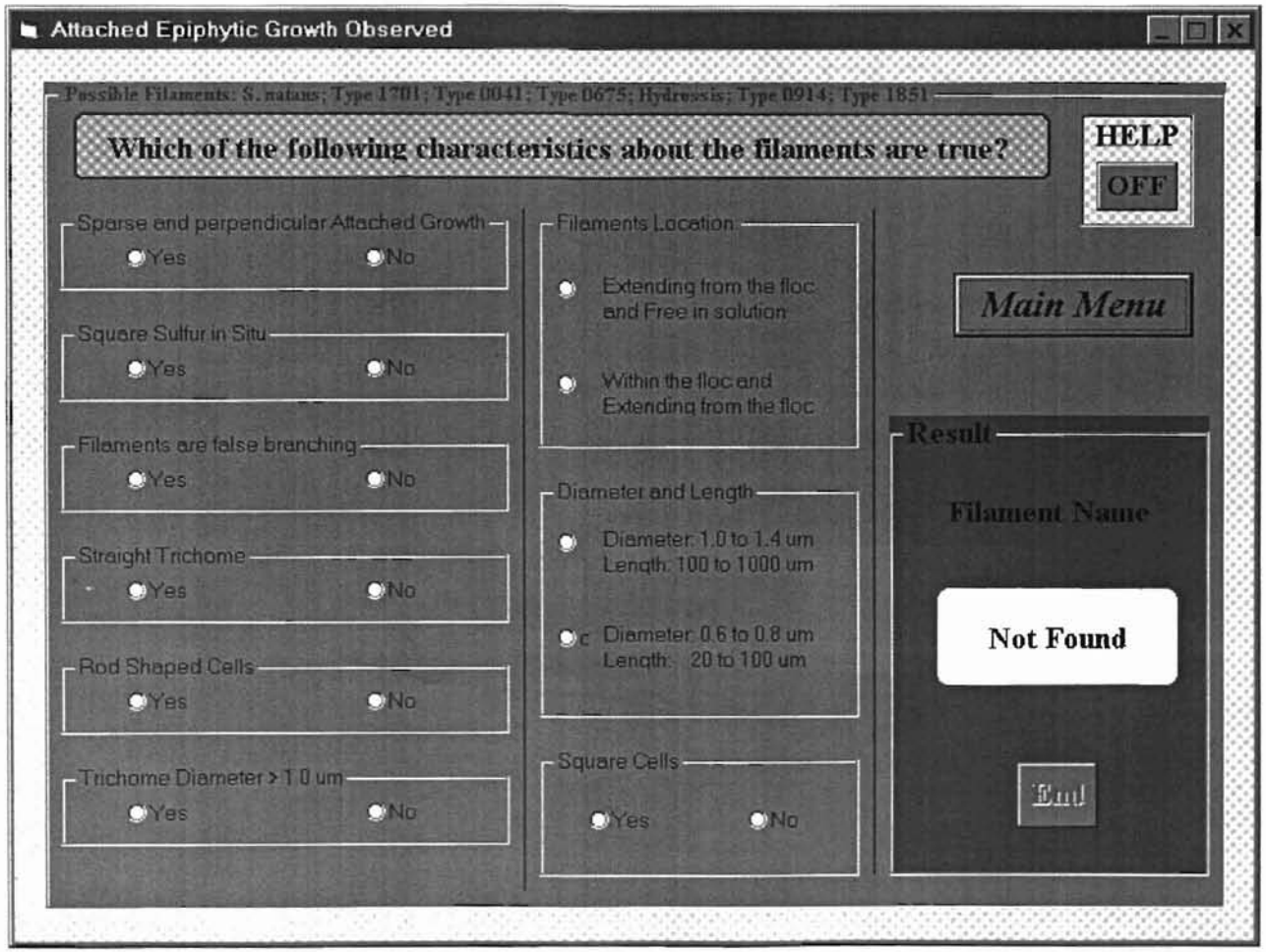

Figure 18. Attached Growth Searching Tree

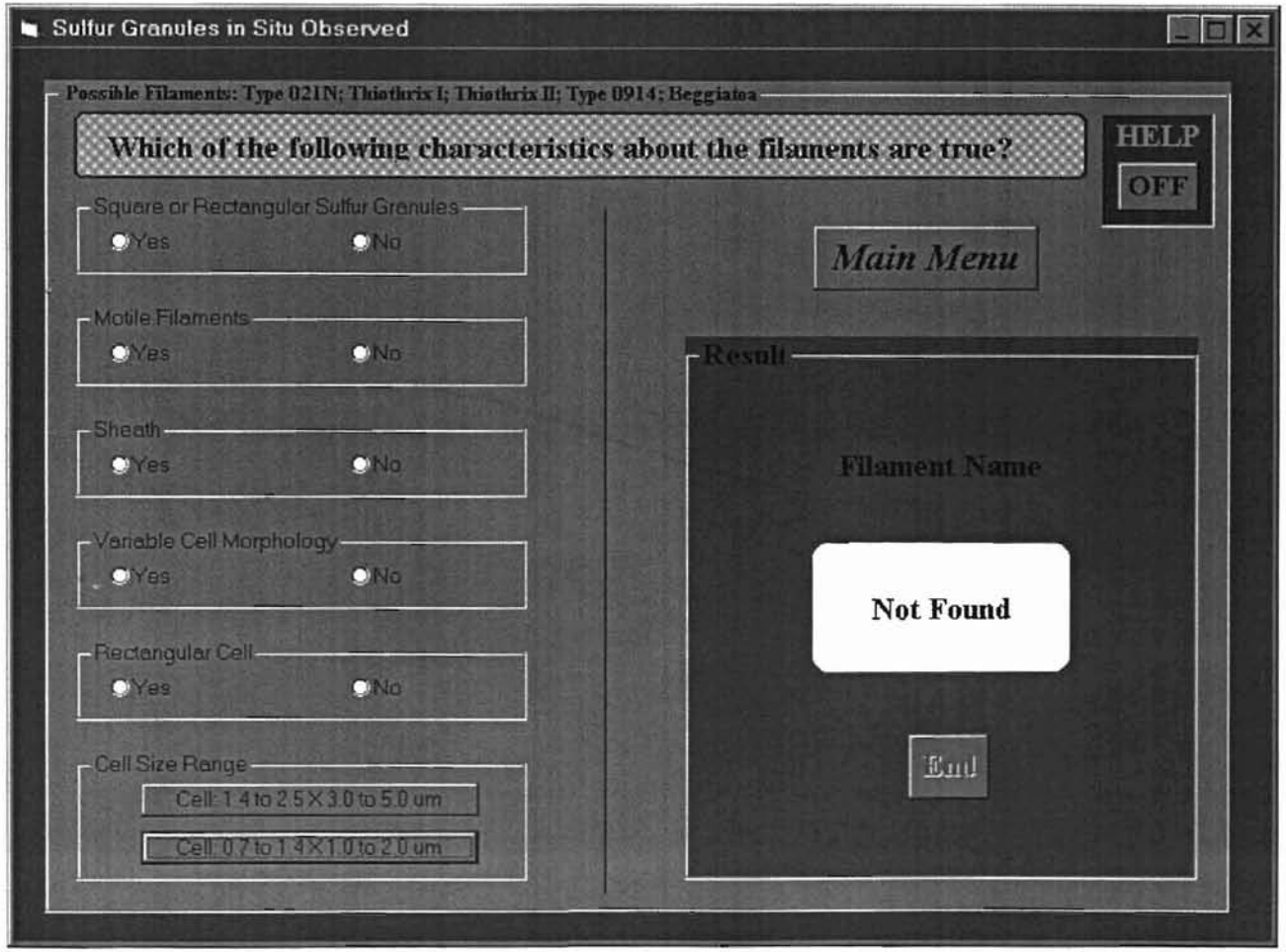

Figure 19. Sulfur Oxidizing Filaments Searching Tree (Page 1)

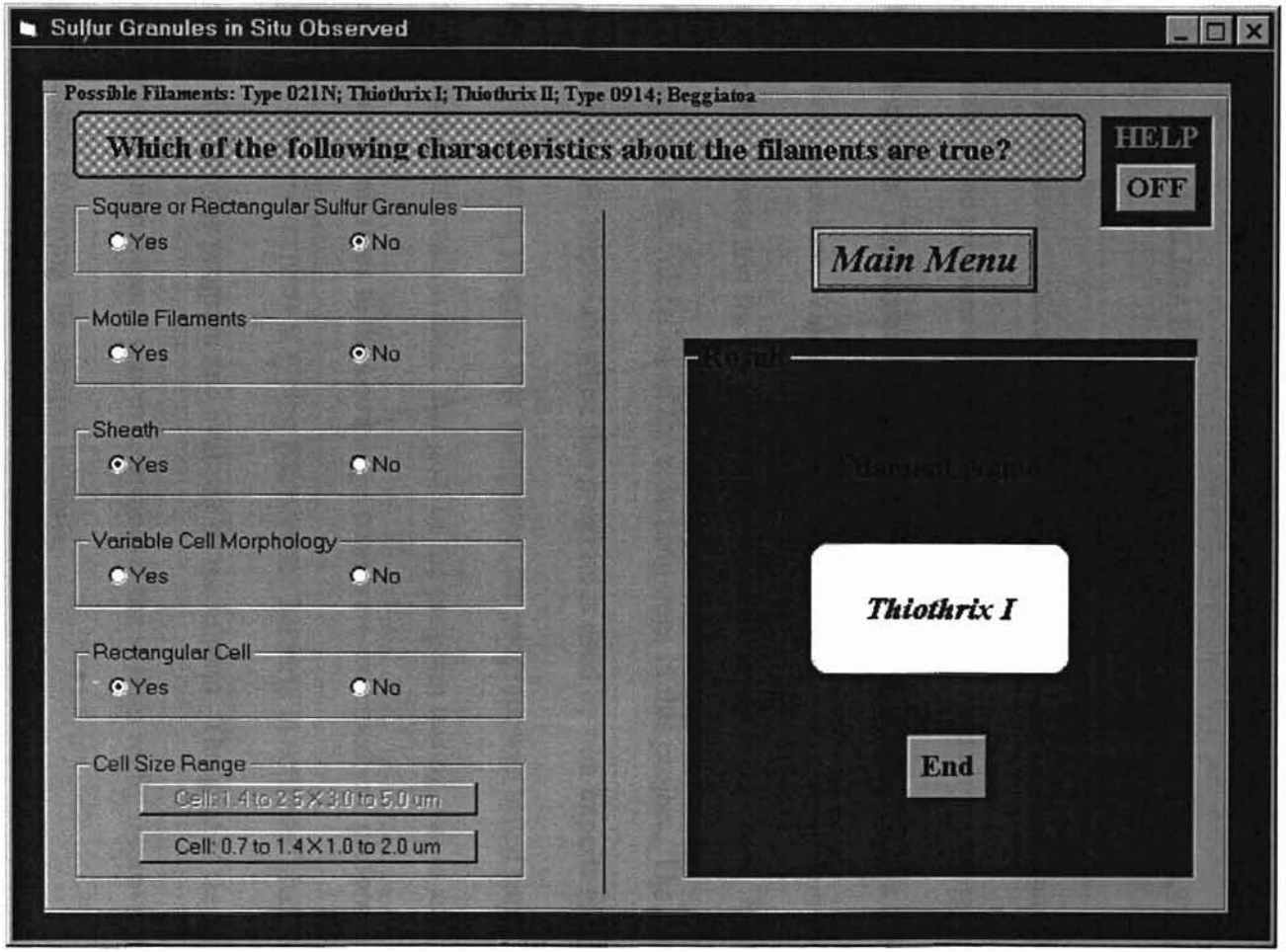

Figure 20. Sulfur Oxidizing Filaments Searching Tree (Page 2)

CHAPTER VII

VALIDATION, SYSTEM TESTING, AND EVALUATION

Program evaluation was the last step in determining if the expert system was successful. The program evaluation included system validation, testing, and evaluation by non-experts. The accuracy of the program was evaluated through a thorough validation procedure. Validation can be defined as building the system so that it elicits the correct conclusion for the actual conditions (Newton et al., 1987). The validation of the program is the validation of the searching trees in this project. The validity of this program is most important when this program is used by non-experts to type filamentous bacteria. After the program was completed, the program was tested using real microscope observation data. The goal of the program testing was to assure that all statements of all rules in the knowledge base were executed and performed as designed. Finally, five non-experts at typing filamentous bacteria in activated sludge samples were selected to run the program and evaluate its usefulness. The goal of the program evaluation by non-experts was to determine if the system really improves human performance in typing filamentous bacteria.

Validation of the Searching Tree

Validation of the searching trees was conducted when the searching trees were constructed. The validation process was accomplished by carefully examining the searching paths and having expert evaluate its correctness and usefulness. Every path of the searching tree was compared with the descriptions provided in the *Manual on the Causes and Control of Activated Sludge Bulking and Foaming* (Jenkins et al., 1993) and *Activated Sludge Microbiology* (Richard, 1989). During this comparison, actual conditions present in typing filamentous bacteria in activated sludge samples were considered based on the writer's typing experiences. These actual conditions mainly included variable characteristics for some filamentous bacteria and some characteristics that are sometimes not easily observed under the microscope. These conditions lead to alternative identification paths in the searching tree for some filamentous bacteria. After validating the trees by comparing each path with those descriptions in Jenkins' manual (Jenkins et al., 1993), an expert at typing filamentous bacteria in activated sludge samples, Chris K. Campana of Stover & Associates, Inc., reviewed these searching trees and provided valuable recommendations.

When validating a path, it was not necessary to match all fifteen characteristics of filamentous bacteria with their descriptions provided by the two manuals. A path was validated based on those special features unique to a specific filamentous organism. For example, *Sphaerotillus natans* can be identified when false branching is present. This searching path can be found in Figure 2. In this path, only one special characteristic, false branching, was used to identify this filamentous bacterium. All special features used in

validating the searching tree were discussed in Chapter V. Also, those special features can be easily observable under 1000X phase contrast.

System Testing

The expert system program and the searching trees were tested during development using previously collected microscope observation data. After the program was completed, it was used routinely to identify filamentous bacteria by the writer at Stover & Associates, Inc. The purpose of the test was to stress, explore, and bracket behavior of the system in a systematic manner. Every item of code and every possible decision was invoked. Every possible outcome of each rule was arrived at.

The program identified nineteen filamentous bacteria successfully during the program testing by using two years microscope observation data from Stover & Associates, Inc. They are listed in Table 4. The program was designed to identify twenty three commonly observed filamentous bacteria in activated sludge processes. Four filamentous bacteria were not identified during the program testing due to no available activated sludge samples containing these four filamentous bacteria. These four filamentous bacteria were Type 0803, 0961, 0411 and *Microthrix parvicella.* Type 0803 can be identified using Figure 4 of the searching tree (Chapter V). Type 0961 is the "transparent" filamentous bacteria which can found in Figure 2. Type 0411 can be identified easily using Figure 2. *M. parvicella* is Gram Stain positive and can be identified using Figure 3.

Table 4

Filamentous Bacteria Identified During Program Development Testing

Filament Name
Sphaerotillus natans
Type 1701
Type 0041
Type 0675
Type 021N
Thiothrix I, II
Type 0914
Beggiatoa spp.
Type 1851
Type 0092
Nocardia spp.
Nostocoida limicola I, II, III
Haliscomenobacter hydrossis
Type 0581
Type 1863
Flexibacter spp.

Evaluation of the Usefulness of the Software by Non-Experts

After the program was developed, an evaluation of the software was conducted utilizing five individuals with vastly different experience at typing filamentous bacteria.

One of them was an engineer working with wastewater treatment processes who had some knowledge of filamentous bacteria typing in activated sludge samples. The remaining four were graduate students majoring in either Environmental Engineering or Environmental Sciences at Oklahoma State University. None of them had any experience in identifying filamentous bacteria in activated sludge samples. Among these four students, one had no microbiology background. Two of the students had limited microbiology exposure. One Ph.D. student had some microbiology background but not in typing filamentous bacteria in activated sludge samples. All five individuals were classified as non-experts in typing filamentous bacteria of a wastewater sample.

The purpose of the evaluation by non-experts was to determine if the system really improved the performance of the individual conducting the typing. One purpose of the project was to assist in filament typing by decreasing the time required to arrive at the correct classification based on the observed characteristics. Essentially, the program accelerates the procedure of working through an identification key. Therefore, the five non-experts used both the software and normal typing procedures provided by manual keys (Jenkins et al., 1993) to identify filamentous bacteria. The speed and the correctness of the software were evaluated by comparing against the normal typing procedure provided by Jenkins et al manual (1993).

Method and Results of Evaluation by Non-experts

Before using the program, each individual was given about 40 minutes of brief introduction to normal typing procedure and a description of this project. They were

asked to do the typing of filamentous bacteria using normal typing procedure first and then using the program to identify the same filamentous bacteria observed under microscope. During the microscopic observation, the writer prepared the slides and adjusted the microscope for them.

Five activated sludge samples were collected from four different wastewater treatment plants for use in this test procedure by non-experts. The filamentous bacteria from these plants have been monitored constantly by Chris K. Campana of Stover & Associates, Inc. Filamentous bacteria identification from three of the five plants has also been confirmed independently by Michael G. Richard of RBD Inc. in Fort Collins, Colorado.

Before the tests by non-experts, the writer identified the true names of the filamentous bacteria in the samples to be used in the tests. These true names are agreed with the identifications made by Chris K. Campana and Michael G. Richard. None of the filamentous bacteria names in the samples were released to the non-experts during these tests.

Seven tests were conducted on these five samples. The times used in observation under the microscope, identification using Jenkins's manual (Jenkins et al., 1993), and identification using the software program were recorded. The results of these tests are compiled in Tables 5 and 6. Table 7 through Table 10 describe all the searching paths in the identification processes.

In Table 5, test number 1 was carried out by non-expert A on sample I. Test number 2 was conducted by non-expert B on sample II. Test number 3 was done by non-expert C on sample III. Samples I, II, and III contained three different types of

filamentous bacteria. Sample I contained *S. natans*, 021N, and 0041. Sample II contained 0041, 021N, and *Thiothrix* I. Sample III contained 0041, *Nocardia* spp., and *Limicola* II. Test number 4 was conducted by non-expert D on sample IV. Test number 5 was conducted by non-expert E on sample IV. Test number 6 was carried out by non-expert D on sample V. Finally, test number 7 was carried out by non-expert E on sample V.

Table 6 summarizes the results of tests 1 through 7. It shows the filaments identified by using the normal typing procedure and the computer program for each test. The times shown in Table 6 are total time used for each test. The real filament names in each test are presented in the last column of Table 6.

Table 5

Filaments Identification Test

Test Number	Non-expert Name	Sample Name
1	A	I
2	B	II
3	C	III
4	D	IV
5	E	IV
6	D	V
7	E	V

Table 6

Evaluation Results of Typing Filamentous Bacteria

Test Number	Microscopic Observation Time (min)	Non-expert Typing Results				Filamentous Bacteria Real Name
		Identification Using Normal Procedure		Identification Using Program		
		Filamentous Bacteria	Time Used (min)	Filamentous Bacteria	Time Used (min)	
1	40	*S. natans*	2	*S. natans*	1	*S. natans*
		021N		021N		021N
		0914		0041		0041
2	40	0914	18	0041	3	0041
		021N		021N		021N
		unknown		*Thiothrix I*		*Thiothrix I*
3	40	0041 or *S. natans*	15	0041	5	0041
		Nocardia spp.		*Nocardia spp.*		*Nocardia spp.*
		Limicola II or 0092		*Limicola II*		*Limicola II*

Table 6 (Continued)

Evaluation Results of Typing Filamentous Bacteria

Test Number	Microscopic Observation Time (min)	Non-expert Typing Results				Filamentous Bacteria Real Name
		Identification Using Normal Procedure		Identification Using Program		
		Filamentous Bacteria	Time Used (min)	Filamentous Bacteria	Time Used (min)	
4	20	*S. natans*	1.39	*S. natans*	1	*S. natans*
5	21	*S. natans*	0.45	*S. natans*	0.47	*S. natans*
6	8	0041	1.37	0041	0.67	0041
7	13	0041	5	0041	3.1	0041

Table 7. Searching Path for Test 1

Searching Path of Normal Typing Procedure	Searching Path of Expert System	Real Name
Filaments do not contain sulfur granules → Gram negative → Cell diameter 1.0 - 2.2 μm → Trichome straight or smoothly curved → Sheath; false branching → ***S. natans***	Figure 4 → Branching → False branching → ***S. Natans***	***S. natans***
Filaments do not contain sulfur granules → Gram negative → Cell diameter 1.0 - 2.2 μm → Trichome straight or smoothly curved → No sheath → Cell barrel or discoid→ **021N**	Figure 5 → Neisser negative → Gram negative → Septa clearly observed → Not rectangular cell → **021N**	**021N**
Filaments do not contain sulfur granules → Gram negative → Cell diameter 1.0 - 2.2 μm → Trichome straight or smoothly curved → No sheath → Cell square → **0914**	Figure 7 → Sparse perpendicular attached growth → Diameter > 1.0 μm → **0041**	**0041**

Table 8. Searching Path for Test 2

Searching Path of Normal Typing Procedure	Searching Path of Expert System	Real Name
Filaments do not contain sulfur granules → Gram negative → Cell diameter 1.0 - 2.2 μm → Trichome straight or smoothly curved → No sheath → Cell square → **0914**	Figure 7 → Sparse perpendicular attached growth → Diameter > 1.0 μm → **0041**	**0041**
Filaments do not contain sulfur granules → Gram negative → Cell diameter 1.0 - 2.2 μm → Trichome straight or smoothly curved → No sheath → Cell barrel or discoid → **021N**	Figure 5 → Neisser negative → Gram negative → septa clearly observed → Not rectangular cell → **021N**	**021N**
Filaments do not contain sulfur granules → Gram negative → Cell diameter 1.0 - 2.2 μm → Trichome straight or smoothly curved → No sheath → Cell rectangular → **0961** → does not match description → **unknown**	Figure 4 → Figure 5 → Figure 6 → Rectangular Cell: (1.4 to 2.5) x (3.0 to 5.0) μm → ***Thiothrix* I**	***Thiothrix* I**

Table 9. Searching Path for Test 3

Searching Path of Normal Typing Procedure	Searching Path of Expert System	Real Name
A. Filaments do not contain sulfur granules → Gram variable or weakly Gram positive → Cell diameter 1.4 - 1.6 μm and heavy attached growth → **0041** B. Filaments do not contain sulfur granules → Gram negative → Cell diameter 1.0 - 2.2 μm and cell septa present → Trichome straight or smoothly curved → sheath; false branching → ***S. natans***	Figure 7 → No sparse & perpendicular attached growth → No square sulfur in situ → No false branching → Not straight filaments → Not rod shaped cells → Trichome diameter > 1.0 μm→ **0041**	**0041**
Filaments do not contain sulfur granules → Gram positive → True branching → ***Nocardia* spp.**	Figure 4 → Branching → True branching → length < 100 μm → ***Nocardia* spp.**	***Nocardia* spp.**
Filaments do not contain sulfur granules → Gram negative → (Cell diameter 1.0 - 1.2 μm → Trichome coiled → ***Limicola* II**) or (Cell diameter < 1.0 μm → Neisser positive → **0092**)	Figure 4 → Neisser stain positive → Not Neisser positive covering → Gram stain negative → Not rectangular cell → Diameter = 1.2 - 1.4 μm → ***Limicola* II**	***Limicola* II**

Table 10. Searching Path for Tests 4, 5, 6, and 7

Searching Path of Normal Typing Procedure	Searching Path of Expert System	Real Name
Filaments do not contain sulfur granules → Gram negative → Cell diameter 1.0 - 2.2 μm → Trichome straight or smoothly curved → Sheath; false branching → ***S. natans***	Figure 4 → Branching → False branching → ***S. Natans***	***S. natans***
Filaments do not contain sulfur granules → Gram variable or weakly Gram positive → Cell diameter 1.4 - 1.6 μm; heavy attached growth presence → **0041**	Figure 7 → Sparse perpendicular attached growth → Diameter > 1.0 μm → **0041**	**0041**

Test 1

Table 7 displays the searching paths utilized in test 1. Test 1 identified three filamentous bacteria. The expert system program identified the three filamentous bacteria correctly. The normal typing procedure failed to identify type 0041 accurately. This was due to the Gram stain variable characteristics of 0041. In the normal typing procedure, 0041 is not in the subtree of Gram negative. The expert system identified 0041 filamentous bacteria using Figure 7, based on attached growth presence on the trichome of filamentous bacteria. This tree is unique from the identification key previously developed by Jenkins et al. (1993).

Test 2

In the second test as shown in Table 8, the expert system identified all three filamentous bacteria correctly while the normal typing procedure failed to identify *Thiothrix* I and type 0041. In the dichotomous key for filamentous organism identification in activated sludge provided by Jenkins et al. (1993), *Thiothrix* I can only be identified when sulfur granules are present on the filament. However, sometimes this filamentous bacteria does not contain any sulfur granules (Jenkins et al., 1993). It is a variable characteristic for *Thriothrix* filamentous bacteria. The expert system program handled this filamentous bacteria by examining all possible filamentous bacteria first and then identified it in Figure 6. Type 0041 can be Gram negative or Gram variable. In the key by Jenkins et al. (1993), 0041 is not in the Gram negative searching key.

Test 3

In the third test as shown in Table 9, all three filamentous bacteria were identified by the expert system correctly. Type 0041 was a filament with attached growth in this case. The expert system identified this filament by examining all the possible filaments with attached growth. While using the normal typing procedure, the non-expert identified two possible filamentous bacteria. This confusion was caused by the Gram stain variable characteristic of 0041. Type 0041 can not be identified under the Gram negative subtree using the procedure set out by Jenkins et al. (1993).

Tests 4 and 5

Tests 4 and 5 were conducted by two non-experts on the same sample. The filamentous bacteria to be identified was *Sphaerotillus natans* (*S. natans*) as shown in the first example of Table 10. *S. natans* was identified correctly by both the normal typing procedure and expert system software. However, the software identified *S. natans* without utilizing Gram stain characteristics by basing its identification solely on the morphological characteristics, namely branching. *S. natans* is fairly easy to identify when false branching can be observed under the microscope. This was one of the design considerations of the searching tree, which was trying to utilize special morphological characteristics of filamentous bacteria to narrow down the possible filaments to be searched.

Test 6 and 7

In tests 6 and 7 type 0041 was identified by two non-experts from the same sample as shown in the second example of Table 10. Both the normal typing procedure and expert system software identified this filament correctly. Again, the software utilized the sparse and perpendicular attached growth characteristics of 0041 to identify it. This increased the speed and confidence of the identification process.

Summary

This chapter discussed the validation of the searching trees developed in this project, system testing, and user evaluation of the software. Validation of the searching trees was accomplished by comparing the developed trees with the descriptions of each filamentous bacteria provided in the *Manual on the Causes and Control of Activated Sludge Bulking and Foaming* (Jenkins et al., 1993) and *Activated Sludge Microbiology* (Richard, 1989) and at the same time taking into consideration the actual conditions when observing under the microscope. After the software was completed, previously collected microscope observation data and routine microscopic identification data from Stover & Associates, Inc. were input into the expert system program. During this system test, the program successfully identified nineteen of the twenty three filamentous bacteria which were designed to be identified by the software. Finally, five individuals with different experience at typing filamentous bacteria evaluated the software by using it to identify the filamentous bacteria in actual wastewater samples.

The evaluation process by users indicated that the expert system provided an excellent method for identifying filamentous bacteria in wastewater samples. The users of the software commented that the software is user friendly and easy to use. The program worked quickly and accurately as indicated by the information in Table 6. Compared to the normal typing procedure, the program which utilized the special morphological characteristics of filamentous bacteria in identification, generally increased the speed and confidence of classification. The program has been used routinely in identifying filamentous bacteria at Stover & Associates, Inc. by the writer.

The complete searching scheme behind the program interface is composed of four major searching trees which are based on special morphological characteristics: neither attached growth nor sulfur granules present, presence of attached growth, presence of sulfur granules in situ, and presence of both attached growth and sulfur granules. Each tree is independent of the others. Inside each tree, filamentous bacteria can be identified using different searching paths. This property of the trees enables the software to handle the variable characteristics of filamentous bacteria easily.

CHAPTER VIII

CONCLUSIONS AND FUTURE WORK

Conclusion

An expert system software has been developed to assist in typing filamentous bacteria in activated sludge samples from wastewater treatment plants. This program is easy to use and can serve as a training program for filamentous bacteria typing.

The program used a set of searching trees developed in this project to identify filamentous bacteria. These searching trees are different from the dichotomous key developed by Jenkins et al. (1993). The filamentous identification key developed in this project consists of four independent trees. Each tree was developed based on the descriptions of filamentous bacteria in *Manual on the Causes and Control of Activated Sludge Bulking and Foaming* (Jenkins et al., 1993), *Activated Sludge Microbiology* (Richard, 1989), and the writer's typing experience. These searching trees allowed multiple identification paths for certain filamentous bacteria depending on what was observed under the microscope. All of these trees were developed based on some special features unique to each filamentous bacteria or to a group of filamentous bacteria.

These searching trees have been validated. Validation was accomplished by comparing each path in the searching tree with the short descriptions of filamentous bacteria provided in *Manual on the Causes and Control of Activated Sludge Bulking and Foaming* (Jenkins et al., 1993) and *Activated Sludge Microbiology* (Richard, 1989). The results of the validation indicated that the trees are accurate. The program has been tested by using previous data and routine filamentous bacteria identification data from Stover & Associates, Inc. Five individuals with different experience at typing filamentous bacteria evaluated the program by using it in actual filamentous bacteria typing. The results indicated that the software worked quickly and accurately. Compared to the normal typing procedure, this preliminary tests by non-experts indicated that the program tended to increase the accuracy and speed of identification through the utilization of the special morphological characteristics of filamentous bacteria.

Future Work

There are several areas where additional work could be performed. One area would be to arrange to test the system in the field using individuals with varying levels of experience in typing filamentous bacteria. This would more thoroughly test the system and probably result in additional enhancements to the system.

Another area where work could be done would include development of a library of filamentous bacteria images. After the program identified filamentous bacteria, the program automatically retrieves the related bacteria images from the image library and projects them on the screen. Also, the image resolution in the program needs to be

improved. Another feature would be to create a data base that would show how often each filamentous bacterium has been identified.

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APPENDIX

FILAMENT DESCRIPTION

Filament Description

Filament Type: *S. natans*

Gram Stain: negative

Neisser Trichome: negative

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: PHB, spherical, often three per cell

Branching: false branching

Motility: no

Filament Shape: straight or smoothly curved

Filament Location: extending from the floc

Filament Diameter: 1.0 to 1.4 µm

Filament Length: 100 to 1000 µm

Attached Growth: no or yes when growing slowly

Sheath: yes and clear, tightly-fitting

Cell Shape: round-ended rods or rectangular when tightly packed in the sheath

Cell Size: 1.0 to 1.8 x 1.5 to 3.0 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: yes

Note: false branching

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 1701

Gram Stain: negative

Neisser Trichome: negative

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: PHB, spherical

Branching: no

Motility: no

Filament Shape: smoothly curved or bent

Filament Location: within the floc and extending from the floc

Filament Diameter: 0.6 to 0.8 µm

Filament Length: 20 to 100 µm

Attached Growth: yes or no when growing rapidly

Sheath: yes and clear, tightly-fitting

Cell Shape: round-ended rod

Cell Size: 0.7 to 1.0 x 1.0 to 2.0 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: yes

Note: cell septa hard to discern

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 0041

Gram Stain: positive or variable

Neisser Trichome: negative

Neisser Granules: negative or positive infrequently

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: none

Branching: no

Motility: no

Filament Shape: straight or smoothly curved

Filament Location: within the floc and extending from the floc

Filament Diameter: 1.4 to 1.6 µm

Filament Length: 100 to 500 µm

Attached Growth: yes (perpendicular), no

Sheath: yes and clear, tightly-fitting

Cell Shape: squares

Cell Size: 1.2 to 1.6 x 1.5 to 2.5 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: no

Note: Neisser stain positive occurs

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 0675

Gram Stain: positive or variable

Neisser Trichome: negative

Neisser Granules: negative or positive infrequently

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: none

Branching: no

Motility: no

Filament Shape: straight or smoothly curved

Filament Location: within the floc

Filament Diameter: 0.8 to 1.0 µm

Filament Length: 50 to 150 µm

Attached Growth: yes, no

Sheath: yes

Cell Shape: squares

Cell Size: 1.0 x 1.0 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: no

Note: Neisser stain positive occurs

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 021N

Gram Stain: negative or positive when contain sulfur granules

Neisser Trichome: negative

Neisser Granules: negative or positive

Sulfur Granules in situ: negative or positive

Sulfur Granules S test: positive or negative

Other Cell Inclusions: PHB

Branching: no

Motility: no

Filament Shape: straight or smoothly curved

Filament Location: extending from floc

Filament Diameter: 1.0 to 2.0 µm

Filament Length: 50 to 1000 µm

Attached Growth: no

Sheath: no

Cell Shape: barrels, rectangles, discoid

Cell Size: 1.0 to 2.0 x 1.0 to 2.0 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: yes

Note: rosettes and gonidia

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: *Thiothrix* I

Gram Stain: negative or positive when contain sulfur granules

Neisser Trichome: negative

Neisser Granules: negative or positive

Sulfur Granules in situ: positive or negative

Sulfur Granules S test: positive

Other Cell Inclusions: PHB

Branching: no

Motility: no

Filament Shape: straight or smoothly curved

Filament Location: extending from floc

Filament Diameter: 1.4 to 2.5µm

Filament Length: 100 to 500 µm

Attached Growth: no

Sheath: yes and heavy

Cell Shape: rectangles

Cell Size: 2.0 x 3.0 to 5.0 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: no

Note: rosettes and gonidia

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: *Thiothrix* II

Gram Stain: negative

Neisser Trichome: negative

Neisser Granules: negative or positive sometimes

Sulfur Granules in situ: positive or negative

Sulfur Granules S test: positive

Other Cell Inclusions: PHB

Branching: no

Motility: no

Filament Shape: straight or smoothly curved

Filament Location: extending from floc

Filament Diameter: 0.7 to 1.4 µm

Filament Length: 50 to 200 µm

Attached Growth: no

Sheath: yes and difficult to detect

Cell Shape: rectangles

Cell Size: 0.7 to 1.4 x 1 to 2 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: no

Note: rosettes and gonidia

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 0914

Gram Stain: negative or positive when contain sulfur granules

Neisser Trichome: negative

Neisser Granules: negative or positive

Sulfur Granules in situ: negative or positive

Sulfur Granules S test: negative

Other Cell Inclusions: PHB

Branching: no

Motility: no

Filament Shape: straight or smoothly curved

Filament Location: extending from floc and free in liquid

Filament Diameter: 0.7 to 1.0 µm

Filament Length: 50 to 200 µm

Attached Growth: no, or incidental

Sheath: no

Cell Shape: squares

Cell Size: 1.0 x 1.0 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: no

Note: sulfur granules "square"

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: *Beggiatoa spp.*

Gram Stain: negative or positive when contain sulfur granules

Neisser Trichome: negative

Neisser Granules: negative or positive

Sulfur Granules in situ: positive or negative

Sulfur Granules S test: positive

Other Cell Inclusions: PHB

Branching: no

Motility: yes

Filament Shape: straight

Filament Location: free in liquid

Filament Diameter: 1.0 to 3.0 µm

Filament Length: 100 to 500 µm

Attached Growth: no

Sheath: no

Cell Shape: rectangles

Cell Size: 1 to 3 x 4 to 8 µm

Crosswalls Clearly Observed: no when sulfur present or yes

Indentations at Crosswalls: no

Note: motile: flexing and gliding

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 1851

Gram Stain: weakly positive and a Gram positive beaded effect

Neisser Trichome: negative

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: none

Branching: no

Motility: no

Filament Shape: straight or smoothly curved

Filament Location: extending from floc

Filament Diameter: 0.8 to 1.0 µm

Filament Length: 100 to 300 µm

Attached Growth: no or yes (perpendicular to the trichome surface)

Sheath: yes and difficult to detect

Cell Shape: rectangles

Cell Size: 0.8 x 1.5 to 2.5 µm

Crosswalls Clearly Observed: yes or no

Indentations at Crosswalls: no

Note: trichome bundles

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 0803

Gram Stain: negative

Neisser Trichome: negative

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: none

Branching: no

Motility: no

Filament Shape: straight or smoothly curved

Filament Location: extending from floc and free in liquid

Filament Diameter: 0.8 µm

Filament Length: 50 to 150 µm

Attached Growth: no

Sheath: no

Cell Shape: rectangles

Cell Size: 0.8 x 1.5 to 2.0 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: no

Note: uniform diameter

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 0092

Gram Stain: negative

Neisser Trichome: positive

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: PHB

Branching: no

Motility: no

Filament Shape: straight and bent

Filament Location: within the floc

Filament Diameter: 0.8 to 1.0 µm

Filament Length: 10 to 60 µm

Attached Growth: no

Sheath: no

Cell Shape: rectangles

Cell Size: 0.8 x 1.5 µm

Crosswalls Clearly Observed: yes or no

Indentations at Crosswalls: no

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 0961

Gram Stain: negative

Neisser Trichome: negative

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: none

Branching: no

Motility: no

Filament Shape: straight

Filament Location: extending from floc

Filament Diameter: 0.8 to 1.4 µm

Filament Length: 20 to 150 µm

Attached Growth: no

Sheath: no

Cell Shape: rectangles

Cell Size: 0.8 to 1.4 x 2.0 to 4.0 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: no

Note: transparent

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: *M. parvicella*

Gram Stain: positive

Neisser Trichome: negative

Neisser Granules: positive

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: PHB

Branching: no

Motility: no

Filament Shape: coiled

Filament Location: within the floc

Filament Diameter: 0.6 to 0.8 µm

Filament Length: 50 to 200 µm

Attached Growth: no

Sheath: no

Crosswalls Clearly Observed: no

Indentations at Crosswalls: no

Note: appear to be large "patches"

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: *Nocardia spp.*

Gram Stain: positive

Neisser Trichome: negative

Neisser Granules: positive

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: PHB

Branching: true branching

Motility: no

Filament Shape: irregularly shaped

Filament Location: within the floc and free in liquid

Filament Diameter: 1.0 µm

Filament Length: 5 to 30 µm

Attached Growth: no

Sheath: no

Cell Shape: variable

Cell Size: 1.0 x 1.0 to 2.0 µm

Crosswalls Clearly Observed: yes or no

Indentations at Crosswalls: no

Note: true branching

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: *N. Limicola* I

Gram Stain: positive or variable

Neisser Trichome: positive

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: none

Branching: no

Motility: no

Filament Shape: coiled and bent

Filament Location: within the floc and free in liquid

Filament Diameter: 0.6 to 0.8 µm

Filament Length: 100 to 200 µm

Attached Growth: no

Sheath: no

Cell Shape: oval when observed

Cell Size: 0.6 x 0.8 µm

Crosswalls Clearly Observed: hard to observe

Indentations at Crosswalls: no

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: *N. Limicola* II

Gram Stain: positive or variable

Neisser Trichome: positive or negative

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: PHB

Branching: no

Motility: no

Filament Shape: coiled and bent

Filament Location: within the floc and extending from the floc

Filament Diameter: 1.2 to 1.4 µm

Filament Length: 100 to 200 µm

Attached Growth: no

Sheath: no

Cell Shape: discs or ovals

Cell Size: 1.2 x 1.0 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: yes

Note: incidental branching

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: *N. Limicola* III

Gram Stain: positive or variable

Neisser Trichome: positive

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: PHB

Branching: no

Motility: no

Filament Shape: coiled and bent

Filament Location: within the floc and extending from the floc

Filament Diameter: 1.6 to 2.0 µm

Filament Length: 200 to 300 µm

Attached Growth: no

Sheath: no

Cell Shape: discs or ovals

Cell Size: 2.0 x 1.5 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: yes

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: *H. hydrossis*

Gram Stain: negative

Neisser Trichome: negative

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: none

Branching: no

Motility: no

Filament Shape: straight and bent, thin filaments

Filament Location: extending from floc and free in liquid

Filament Diameter: 0.5 μm

Filament Length: 10 to 100 μm

Attached Growth: no or yes, attached growth are common

Sheath: yes

Crosswalls Clearly Observed: no

Indentations at Crosswalls: no

Note: rigidly straight

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 0581

Gram Stain: negative

Neisser Trichome: negative

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: none

Branching: no

Motility: no

Filament Shape: coiled

Filament Location: within the floc or patches free in liquid

Filament Diameter: 0.4 to 0.7 µm

Filament Length: 100 to 200 µm

Attached Growth: no

Sheath: no

Crosswalls Clearly Observed: no

Indentations at Crosswalls: no

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 1683

Gram Stain: negative

Neisser Trichome: negative

Neisser Granules: negative or positive

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: none

Branching: no

Motility: no

Filament Shape: bent and irregularly shaped, short

Filament Location: extending from floc and free in liquid

Filament Diameter: 0.8 µm

Filament Length: 20 to 50 µm

Attached Growth: no

Sheath: no

Cell Shape: oval rods

Cell Size: 0.8 x 1.0 to 1.5 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: yes

Note: "Chain of cells"

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

Filament Description (Continued)

Filament Type: Type 0411

Gram Stain: negative

Neisser Trichome: negative

Neisser Granules: negative

Sulfur Granules in situ: negative

Sulfur Granules S test: negative

Other Cell Inclusions: none

Branching: no

Motility: no

Filament Shape: bent and irregularly shaped

Filament Location: extending from floc

Filament Diameter: 0.8 µm

Filament Length: 50 to 150 µm

Attached Growth: no

Sheath: no

Cell Shape: elongated rods

Cell Size: 0.8 x 2.0 to 4.0 µm

Crosswalls Clearly Observed: yes

Indentations at Crosswalls: yes

Note: "Chain of cells"

Source: i Jenkins, D., Richard, M. G. and Daigger, G. T., 1993. *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Lewis Publishers.

Source: ii Richard, Michael, Ph.D., 1989. *The Bench Sheet Monograph on Activated Sludge Microbiology*, The Water Pollution Control Federation.

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