Microscopy of Activated Sludge

Presented By:

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Activated Sludge Introduction

Eikelboom, D.H. (2000). *Process Control of Activated Sludge Plants by Microscopic Investigation.* London, UK: IWA Publishing.

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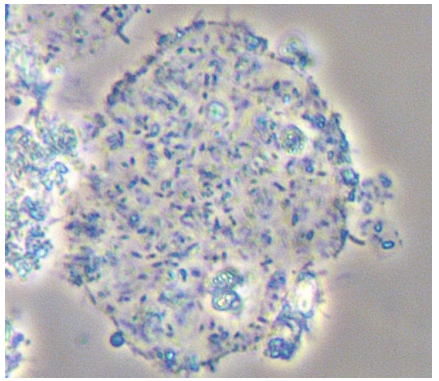
Activated Sludge

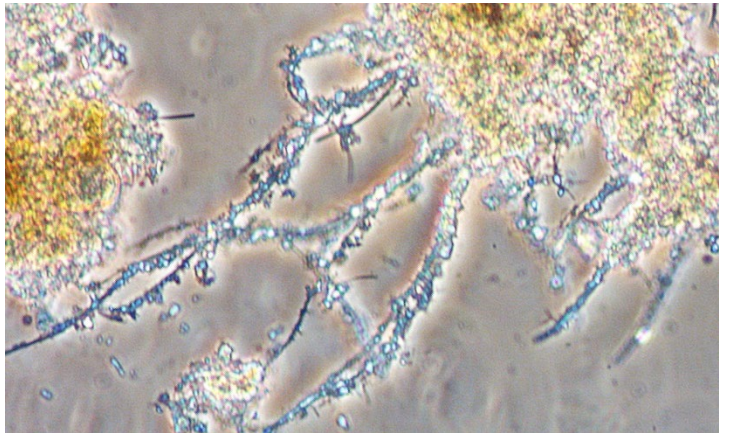
- The activated sludge process is used to treat wastewater.
- It consists of flocs made up of organic/inorganic material and microorganisms.
- Microorganisms, such as bacteria, protozoans, and metazoans, consume and remove the waste from the wastewater.

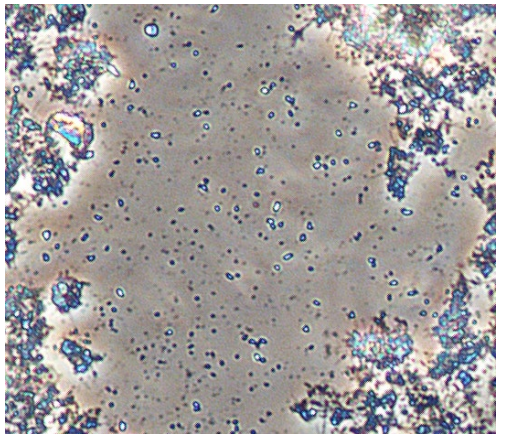
https://www.watertechonline.com/wastewater/article/15550311/aerated-activated-sludge-basics



Activated Sludge Biomass

- The composition of a floc's biomass can indicate issues with WWTP operations
  - Filamentous bacteria - cause sludge bulking and foaming
  - Spirils - indicate lack of oxygen
  - Excess free-living cells - do not settle in the final clarifier, negatively impacting final effluent quality
- Microscopic investigation helps identify issues caused by biomass composition





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Microscope Basics

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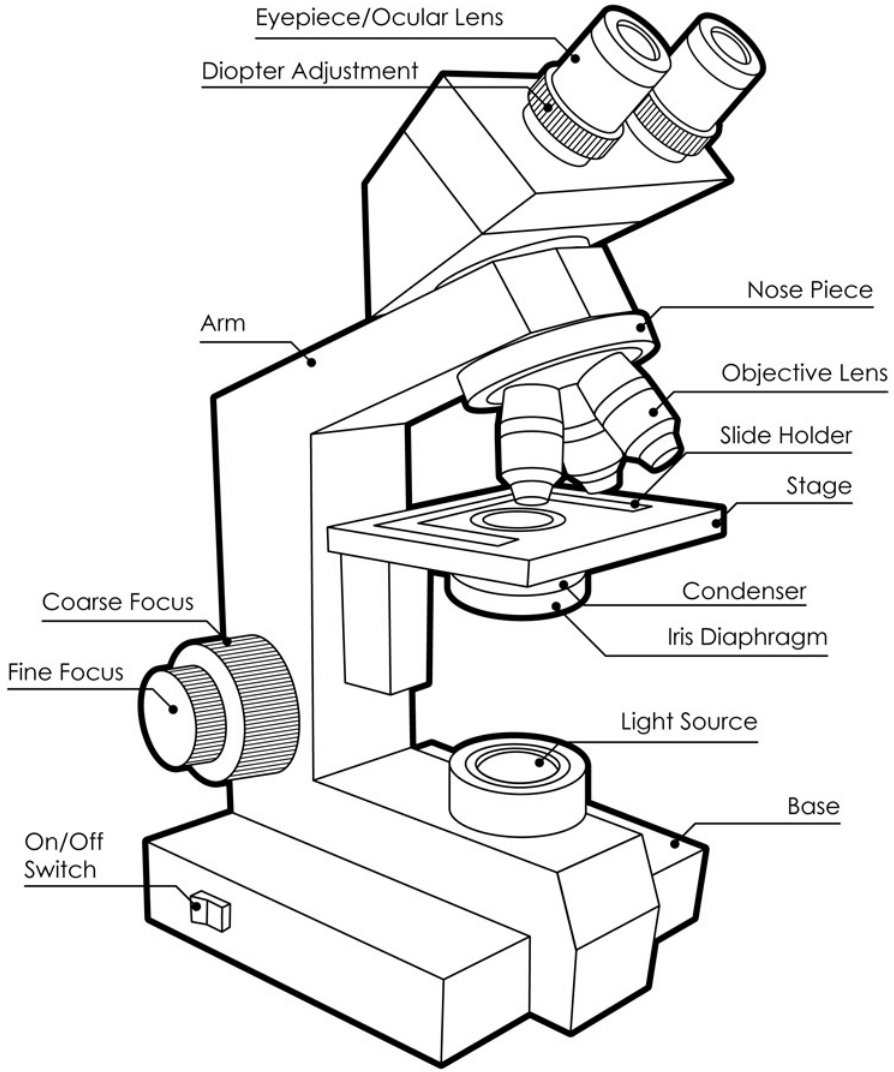
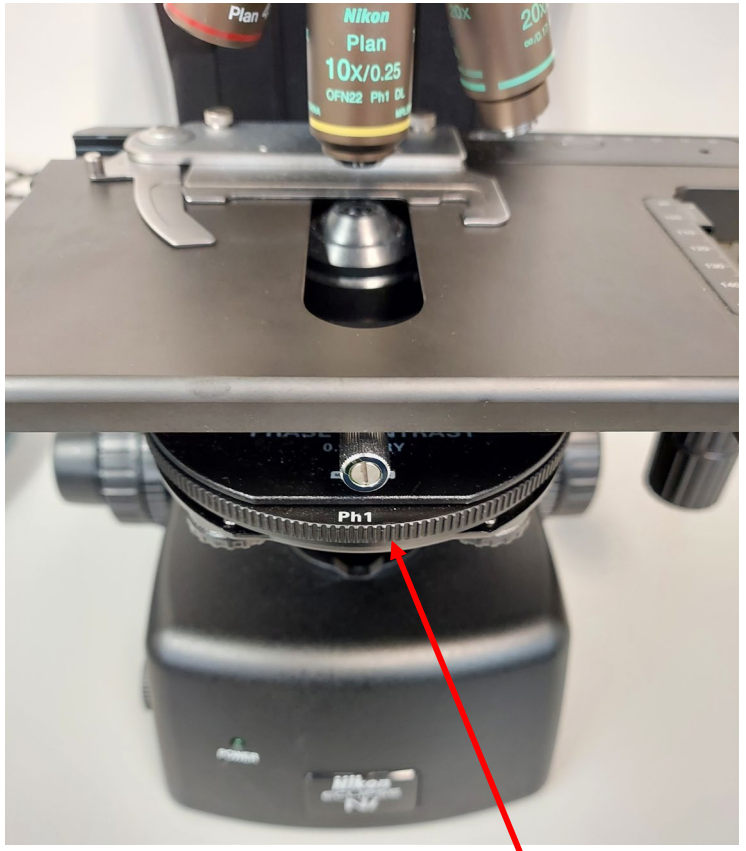
Parts of a Microscope



https://line.17qq.com/articles/cwessaahx.html



Phase condenser

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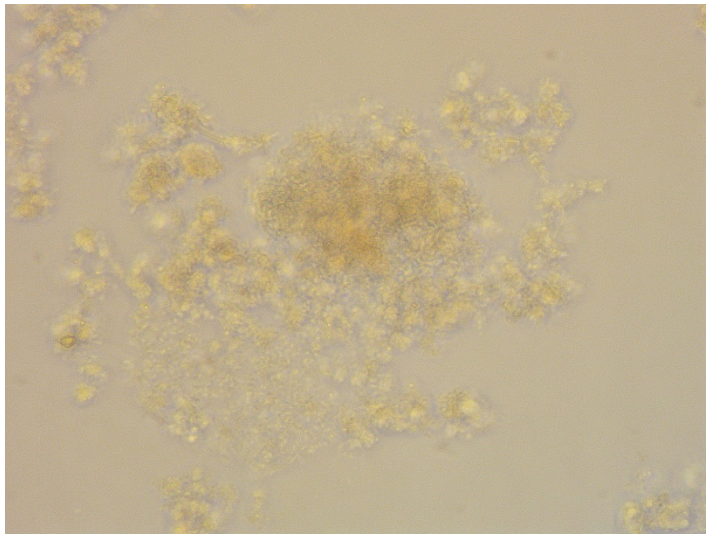
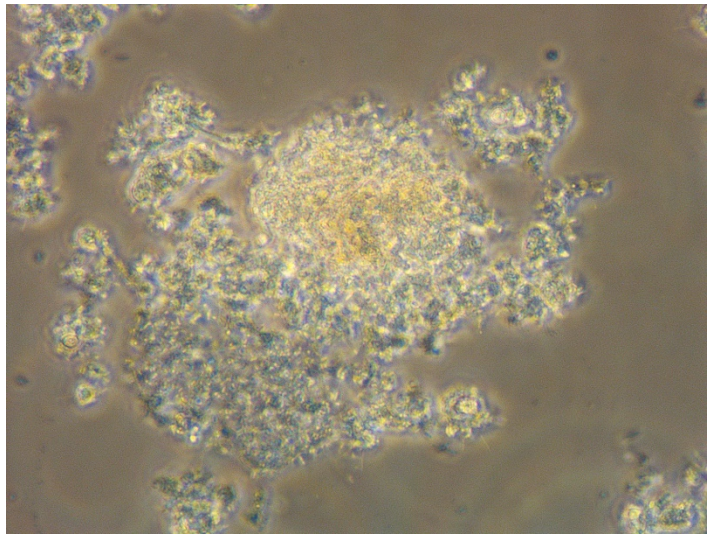
Microscope Use

- Activated sludge has low contrast
  - Light microscopes with phase contrast should be used to distinguish small differences in the floc

Bright Light



Phase Contrast



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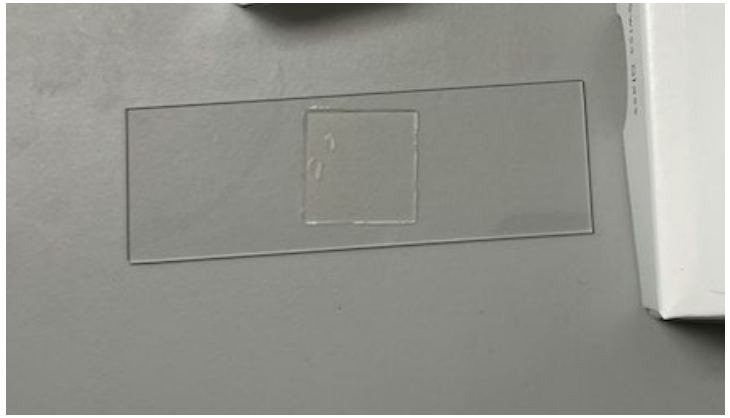
Microscope Schematics

Total Magnification:
10x * 20x = **200x**

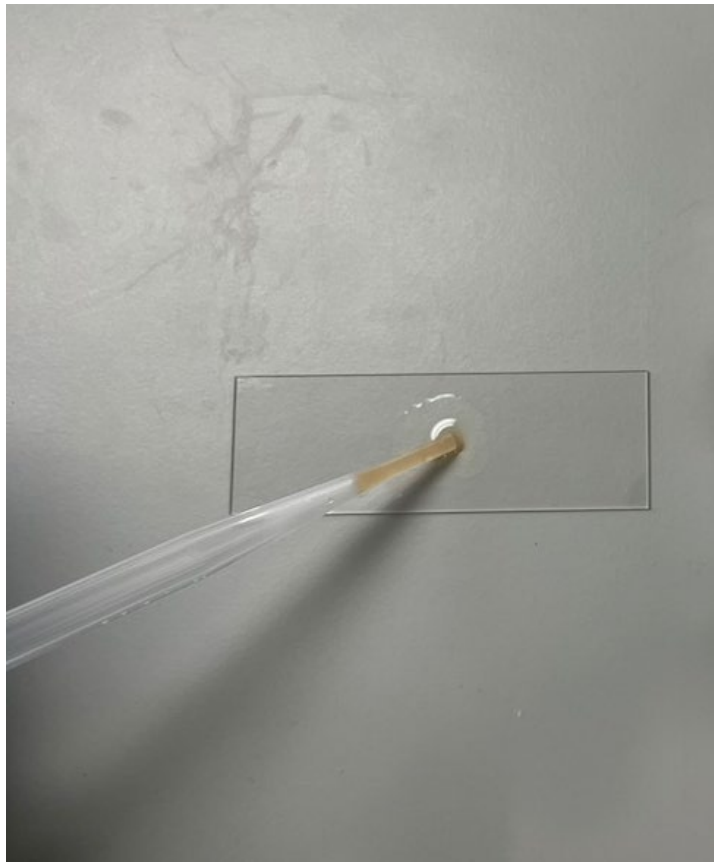
- Magnification is two-fold
  - The eyepiece
    - Usually 10x, but can be 6x, 15x, or 20x
  - The objective (lens)
    - Usually 3 or 4 objectives at 10x, 20x, 40x, and 100x
  - Eyepiece x Objective = Total magnification
- High magnifications (e.g. 100x objective) require immersion oil to be used between the objective lens and cover slip

Slide Preparation – Wet Mount



- Place a drop of the sludge on a glass side, then cover with a cover slip.
- Try to avoid trapping air bubbles under the slip.

Slide Preparation – Fixed Smear



- Place a small drop on the slide.
- Spread drop over the surface of the slide.
- Let slide air dry.

Staining Procedures

Eikelboom, D.H. (2000). *Process Control of Activated Sludge Plants by Microscopic Investigation.* London, UK: IWA Publishing.

Water Environment Federation (2001). *Wastewater Biology: The Microlife.* Alexandria, VA: Water Environment Federation.

Van Dommelen, J. (2019). *Staining Techniques: Revealing the Hidden*. 2019 Troubleshooting Activated Sludge. OWEA Advanced Activated Sludge Workshop.

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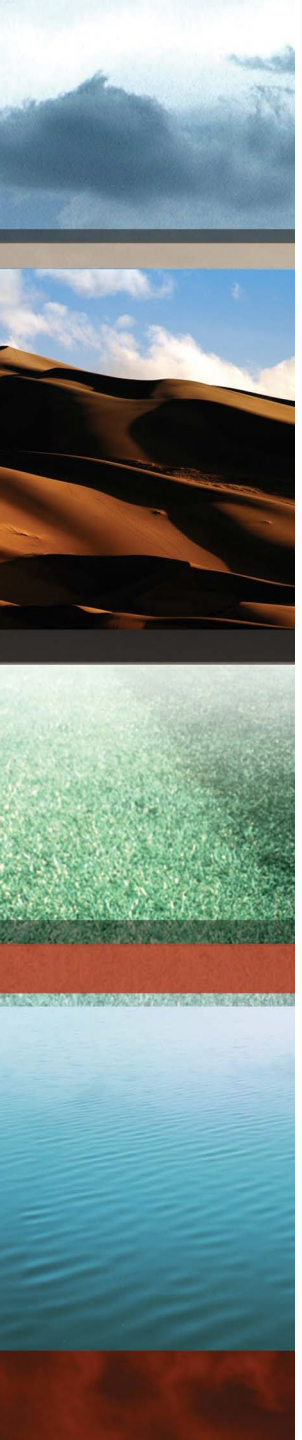

Staining Procedures

- Two staining procedures aid in the identification of filamentous bacteria:
  - Gram staining
  - Neisser staining
- India ink reverse staining:
  - Allows plants to identify if their activated sludge contains excess extracellular polymeric substances (EPS)
  - Large amounts of EPS can produce non-filamentous viscous bulking, reduced ability to flow, and can cause solids dewatering issues

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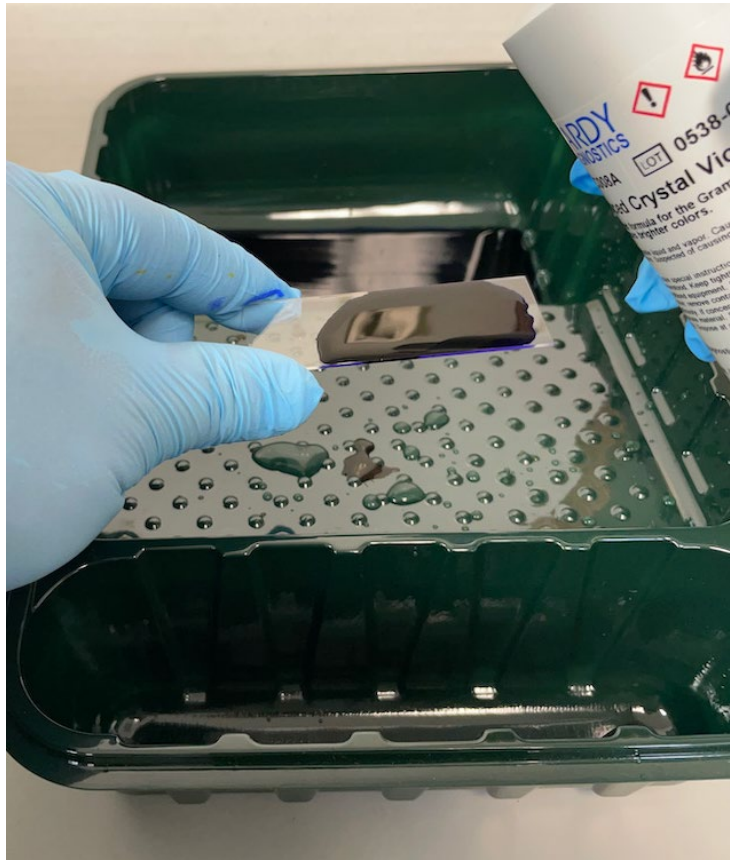
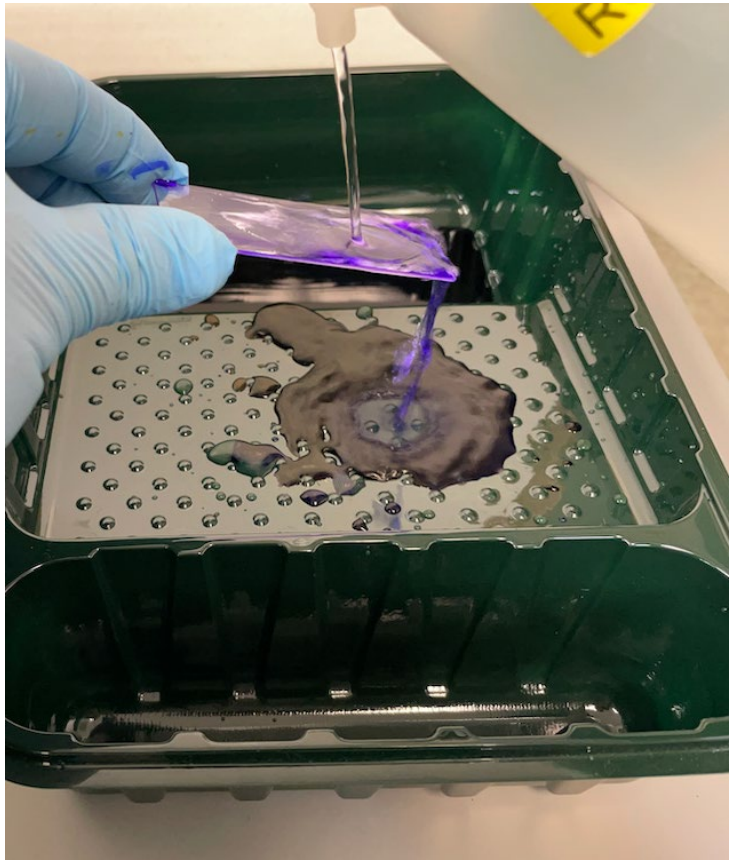
Gram Staining

- Prepare a fixed smear slide
- The following are needed:
  - Crystal Violet Solution
  - Iodine Solution
  - Decolorizing Solution
  - Safranin Solution
    (counterstain)

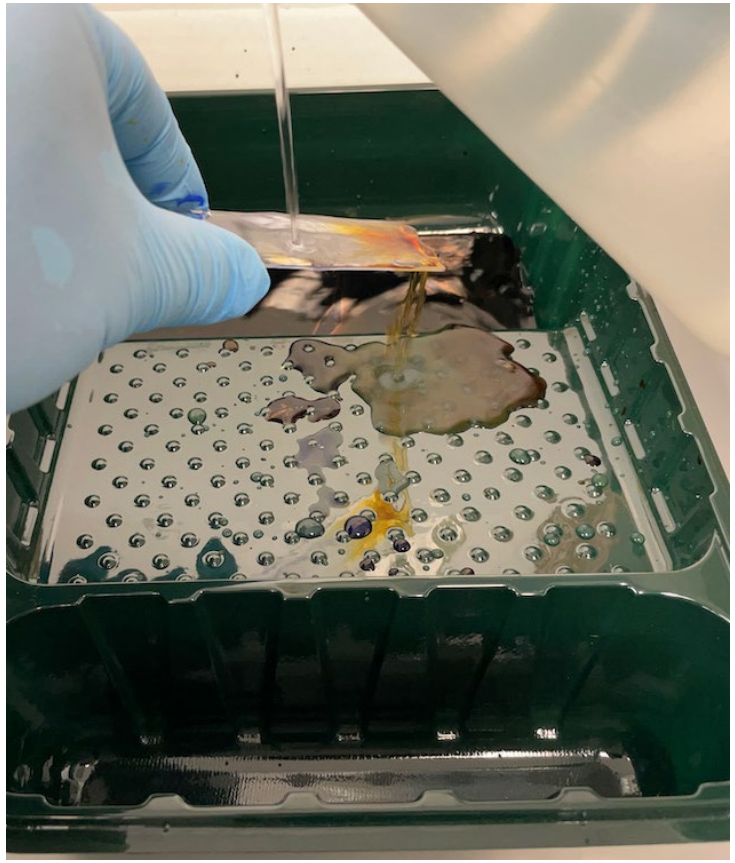
Gram Staining – Step 1





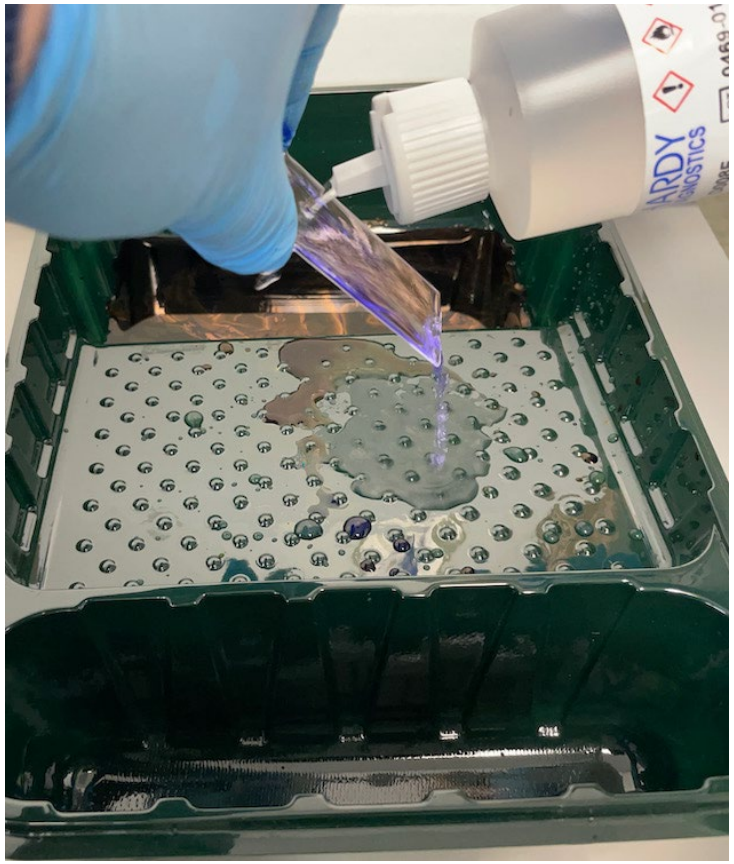
- Crystal Violet
  - Flood slide for 1 minute
  - Rinse with DI water

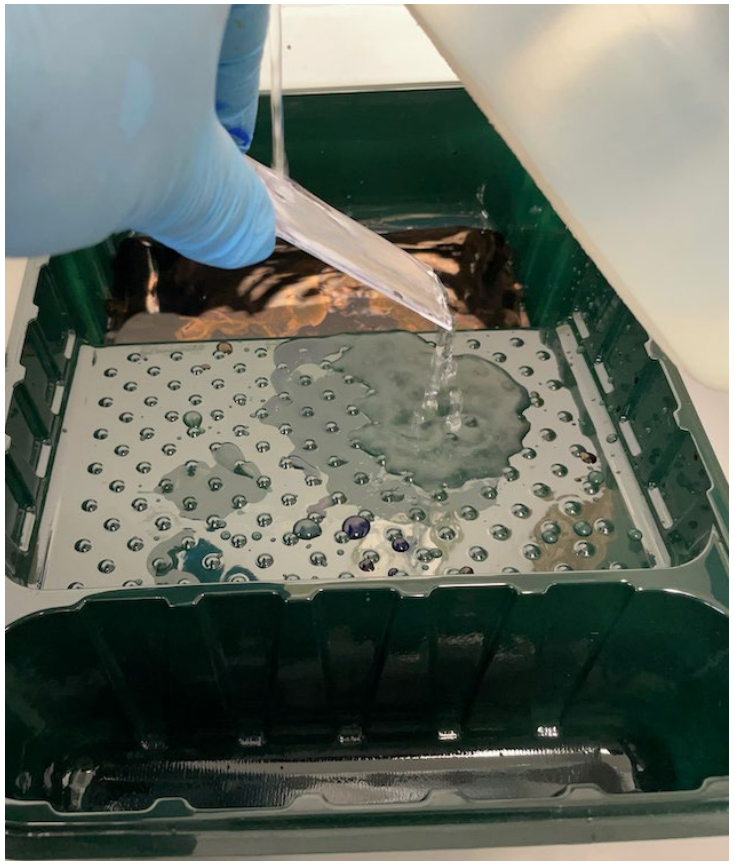
Gram Staining – Step 2



- Iodine Solution
  - Flood slide for 1 minute
  - Rinse with DI water

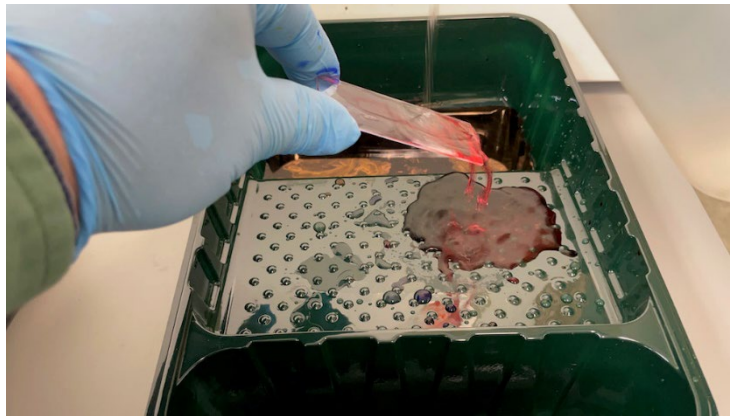
Gram Staining – Step 3

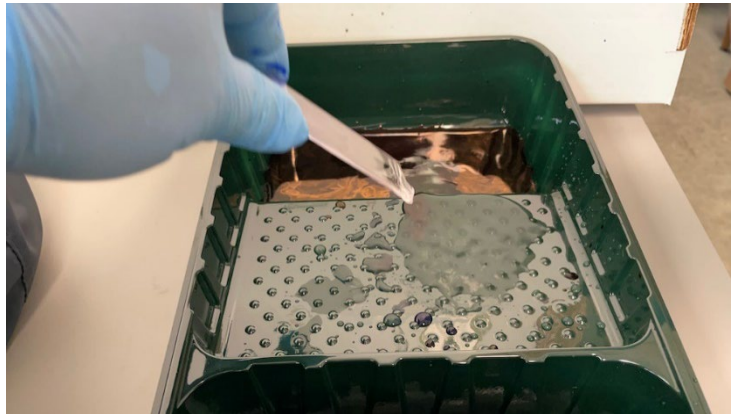




- Decolorizing Solution
  - Hold slide at 45 degrees
  - Apply dropwise until blue color stops rinsing off
  - Blast with DI water to stop reaction
  - Blot dry with paper towel

Gram Staining - Step 4

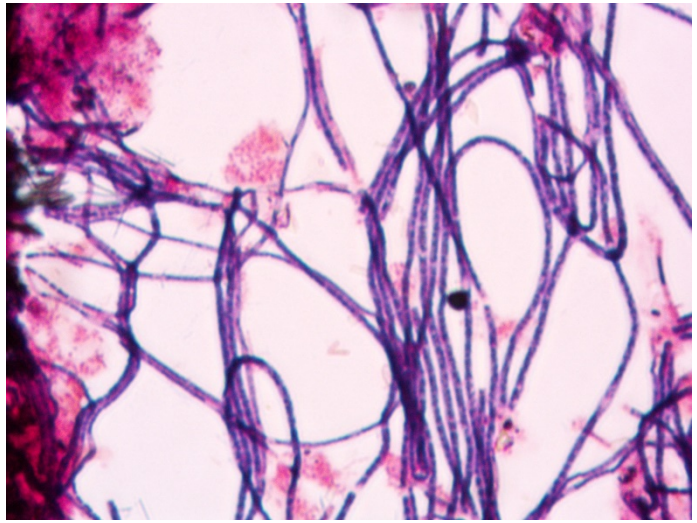




- Safranin Solution
  - Flood slide for 1 minute
  - Rinse with DI water
  - Let air dry

Gram Staining – Step 5

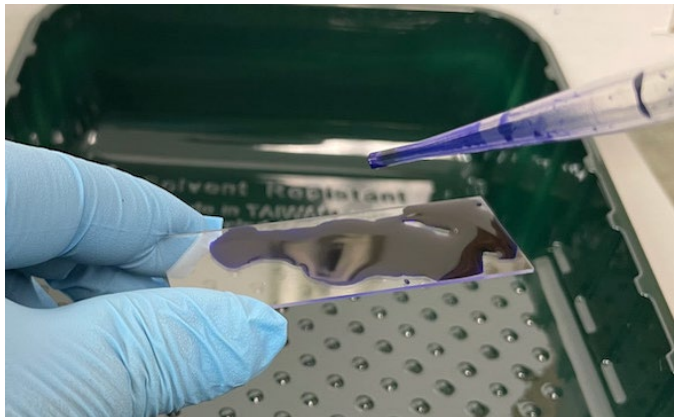
- View slide at 1000x (using oil immersion) under bright light
- Gram negative
  - Stains red
  - Bacteria release the absorbed blue dye during step 3 (decolorizing)
- Gram positive
  - Stains blue
  - Bacteria do not release the blue dye during step 3 (decolorizing)

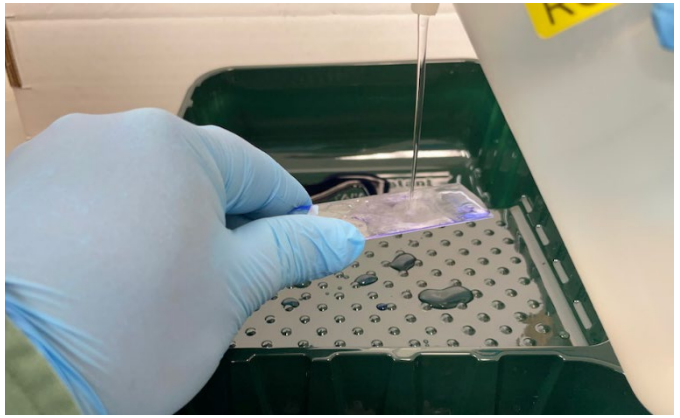


Neisser Staining

- Prepare a fixed smear slide
- The following are needed:
  - Methylene blue solution
  - Crystal violet solution
  - Bismark brown solution

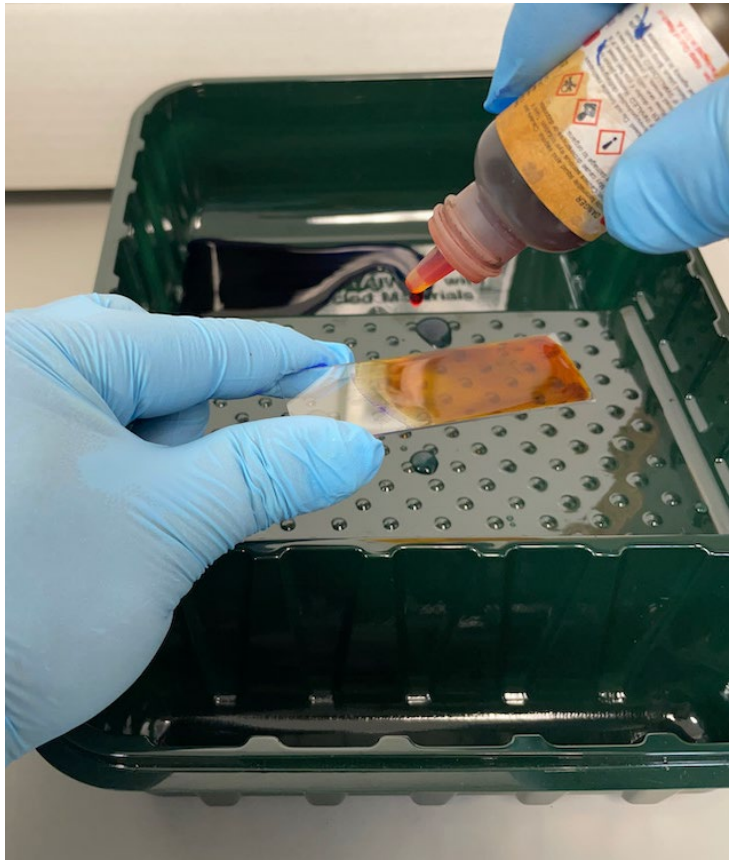
Neisser Staining – Step 1

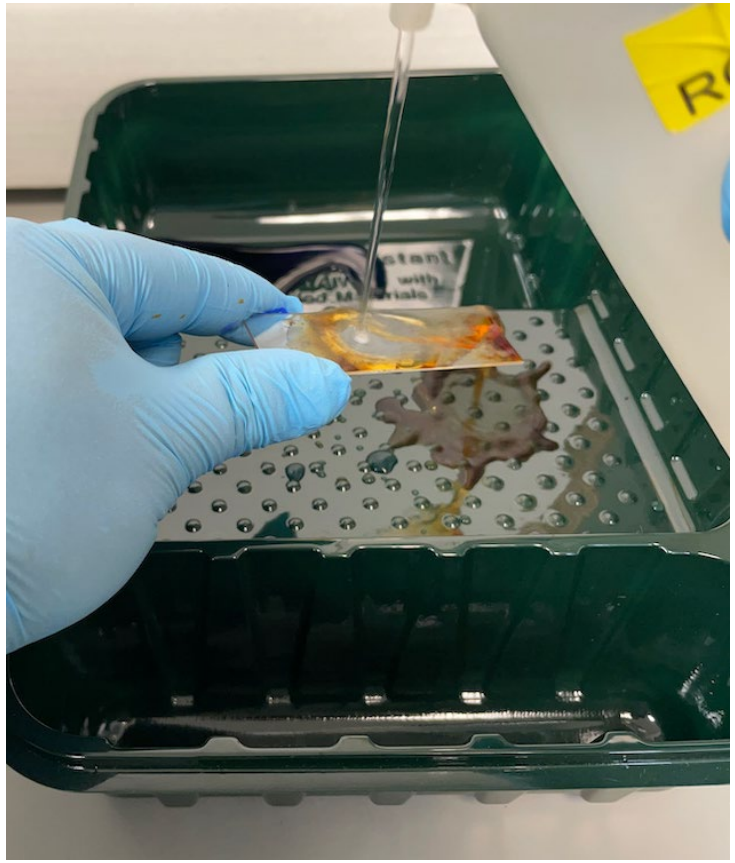




- Mix 2 parts Methylene Blue and 1 part Crystal Violet in a small container
- Flood slide for 30 seconds
- Rinse with DI water

Neisser Staining – Step 2

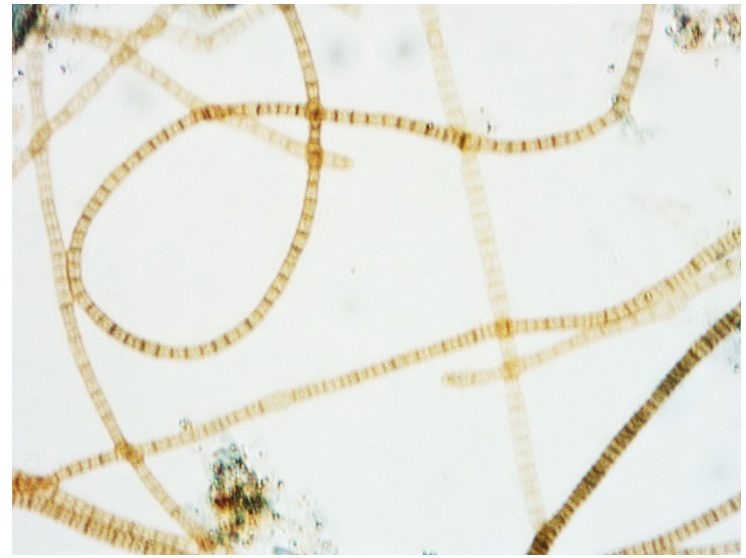




- Bismark Brown Solution:
  - Flood slide for 1 minute
  - Rinse with DI water
  - Blot dry (do not rub)

Neisser Staining – Step 3

- View slide at 1000x (using immersion oil) under bright light
- Neisser negative
  - Slightly brown or yellow
- Neisser positive
  - Indicates presence of polyphosphates stored in cells
  - 3 types:
    1. Completely gray-violet
    2. Blue-black colored globules
    3. Colonies of blue-black cells



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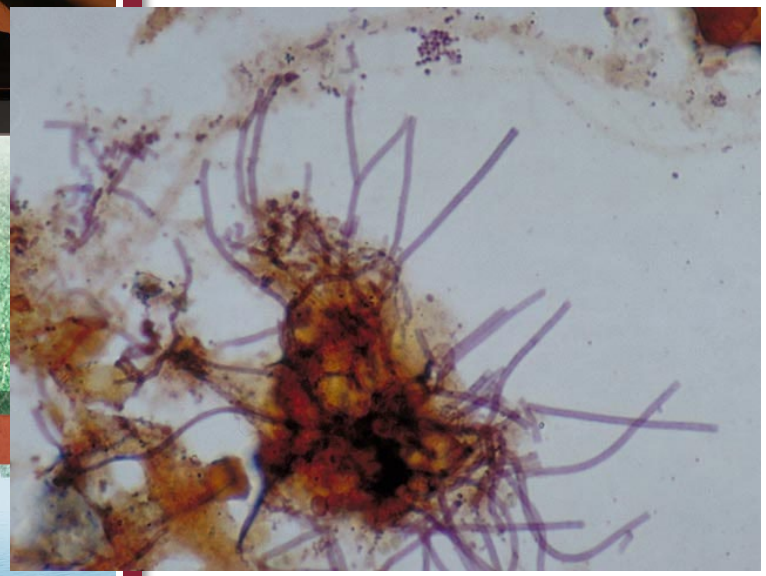
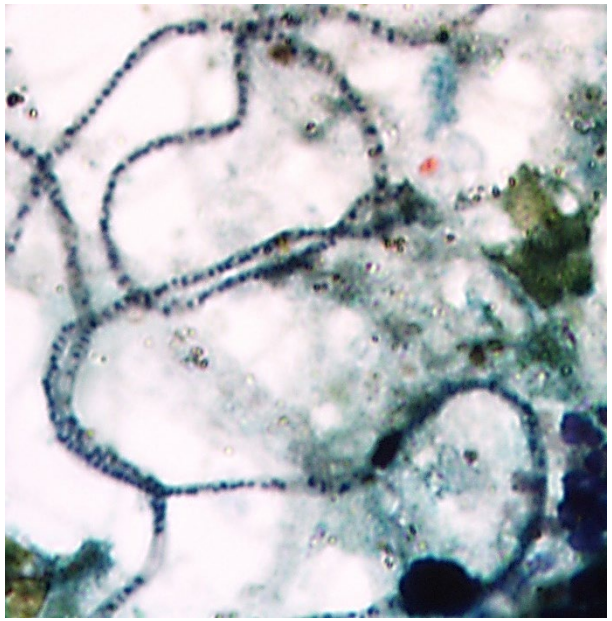
Neisser Staining – Neisser Positive

Gray-violet

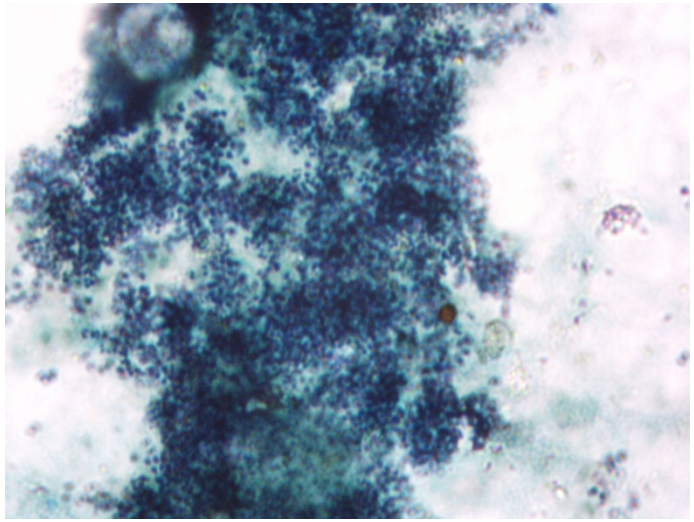


https://www.asissludge.com/NeisGram.htm

Blue-black colored globules



Colonies of blue-black cells



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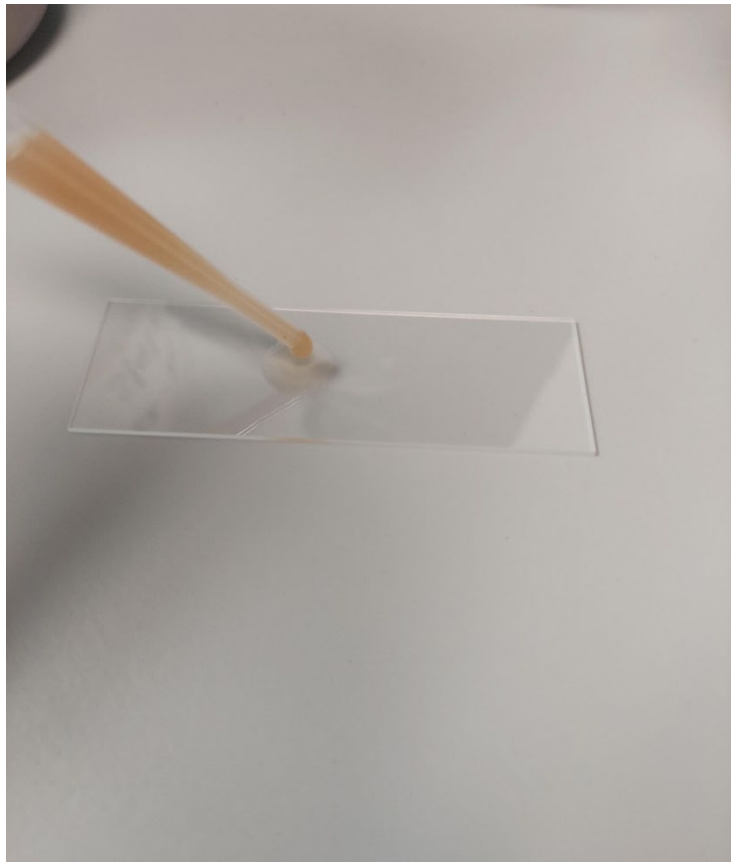
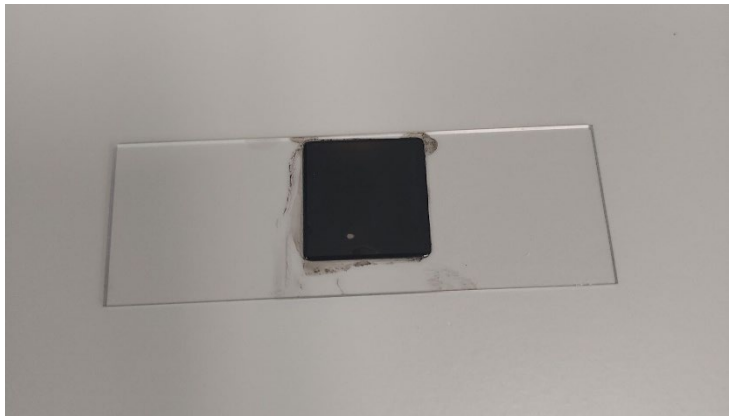
India Ink Reverse Staining

- Prepare a wet mount slide
- The following is needed:
  - India Ink

India Ink Reverse Staining – Step 1





- Mix 1 drop of activated sludge and 1 drop of India ink on slide
- Place cover slip on slide

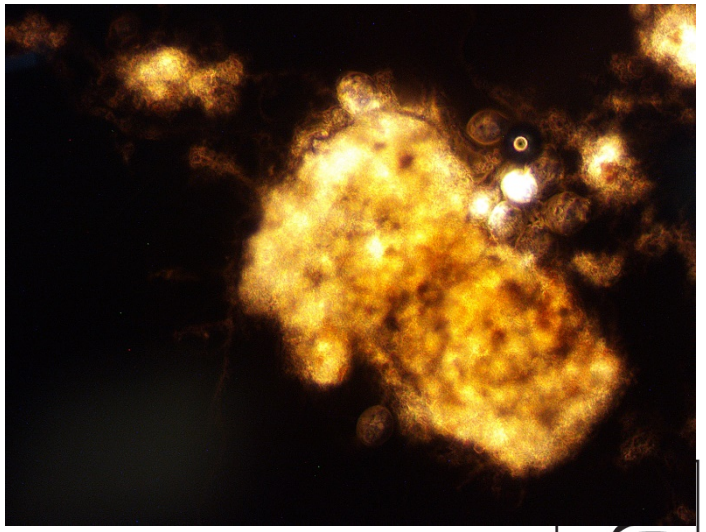
India Ink Reverse Staining – Step 2

- Observe under phase contrast
- In normal sludge, the ink should penetrate the flocs almost completely, though may leave some clear centers
- In sludge with excess EPS, the ink does not penetrate the floc

Less EPS

https://www.biologicalwasteexpert.com/blog/india-ink-test-an-easy-way-to-monitor-floc-extracellular-polymers

More EPS



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Filamentous Microorganism Identification

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Water Environment Federation (2001). *Wastewater Biology: The Microlife.* Alexandria, VA: Water Environment Federation.

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Filamentous Organisms

- Filaments are normal occurrences in activated sludge and contribute to the treatment process
- Filaments can be bacteria, fungi, and algae
- Filaments form when:
  - The cells do not detach from each other after cell division, or
  - Detachment is impossible due to the presence of a sheath

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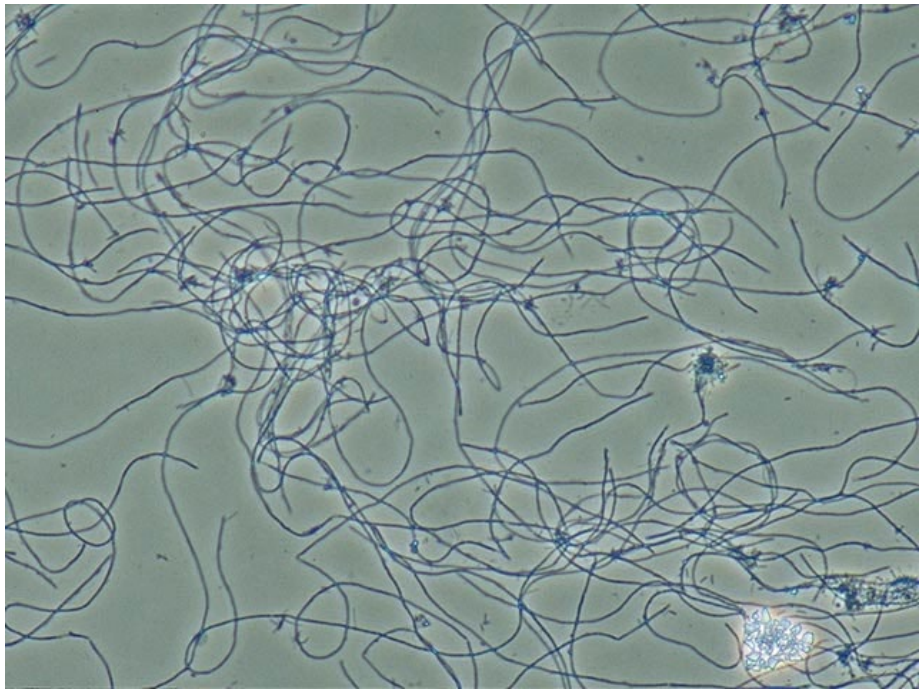
Filamentous Organisms

- Excess filaments can cause:
  - Bulking
  - Deterioration of settling and dewatering properties of the sludge, and
  - Scum formation
- Identification of filamentous organisms can aid in troubleshooting and resolving process conditions
- Methods to control sludge issues can be dependent upon which filamentous species is present in the sludge
    - E.g. the presence of *Microthrix parvicella*, a filament that causes bulking and scum formation, can often be controlled by dosing with Aluminum salts



https://www.asissludge.com/Scripts/F22211210.htm

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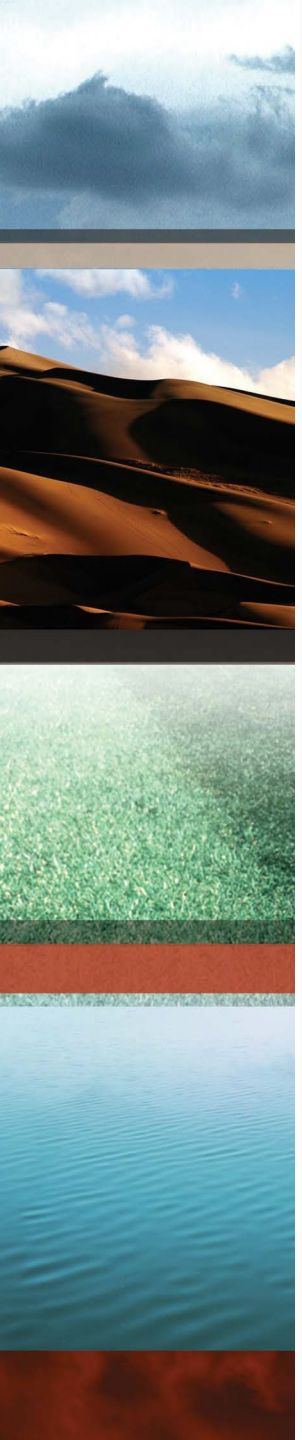

Filamentous Identification

- 11 Characteristics are used to identify filamentous microorganisms
  1. Mobility
  2. Branching
  3. Filament Shape
  4. Attached growth
  5. Filament diameter
  6. Septa or transverse walls
  7. Cell shape
  8. Sheath
  9. Granules
  10. Gram staining results
  11. Neisser staining results

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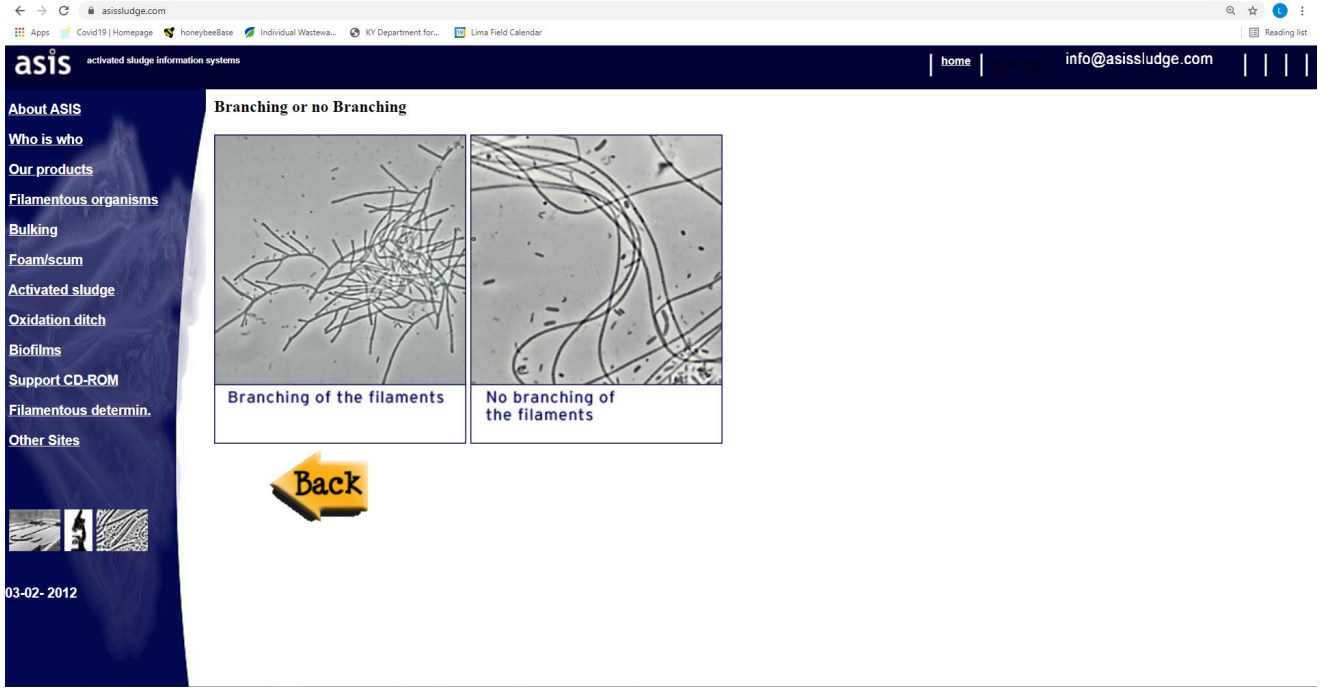
Filamentous Identification

- There are several tables and flow charts that can be used to aid in filamentous identification
  - The next 3 slides are tables and flow charts discussed in the book *Process Control of Activated Sludge Plants by Microscopic Investigation* (Eikelboom, 2000).
- https://www.asissludge.com/ also provides a free interactive identification key



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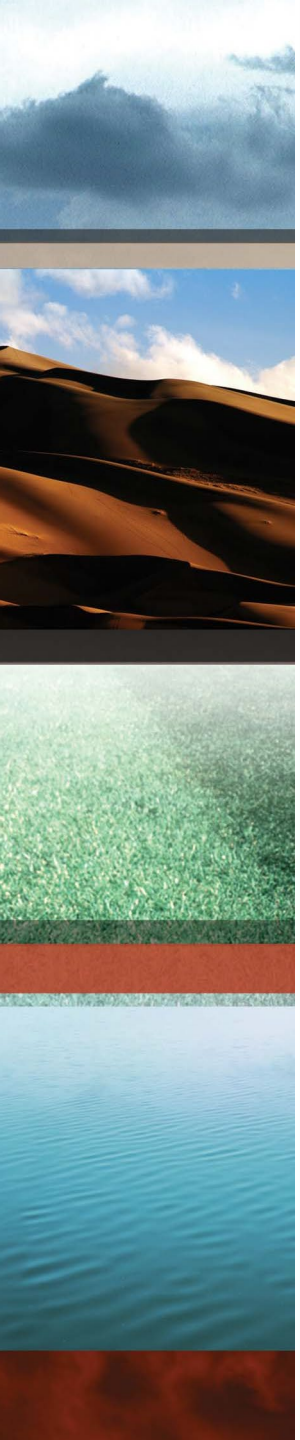
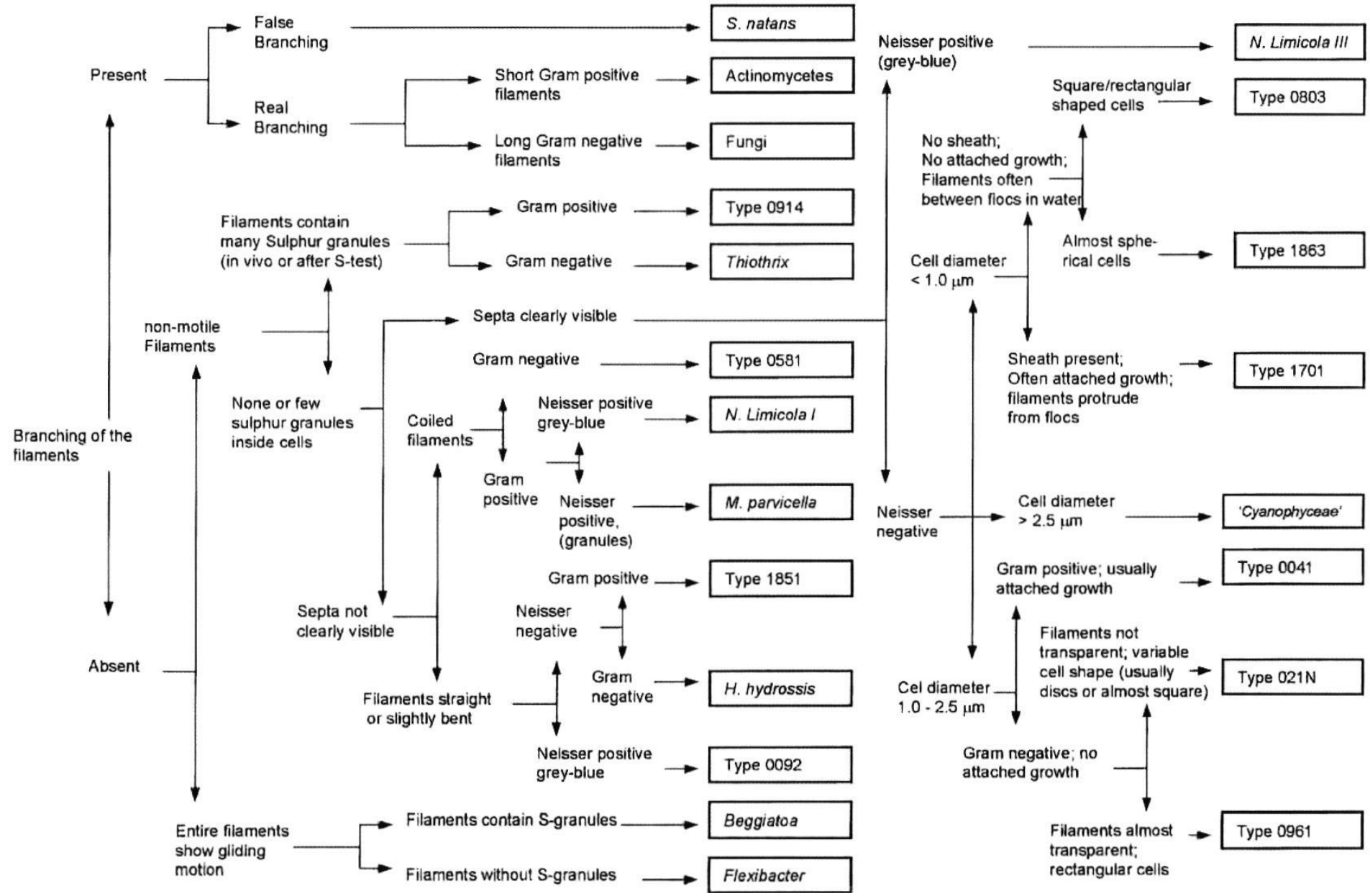


Table 1 *Characteristics of the filamentous micro-organisms included in the identification key*

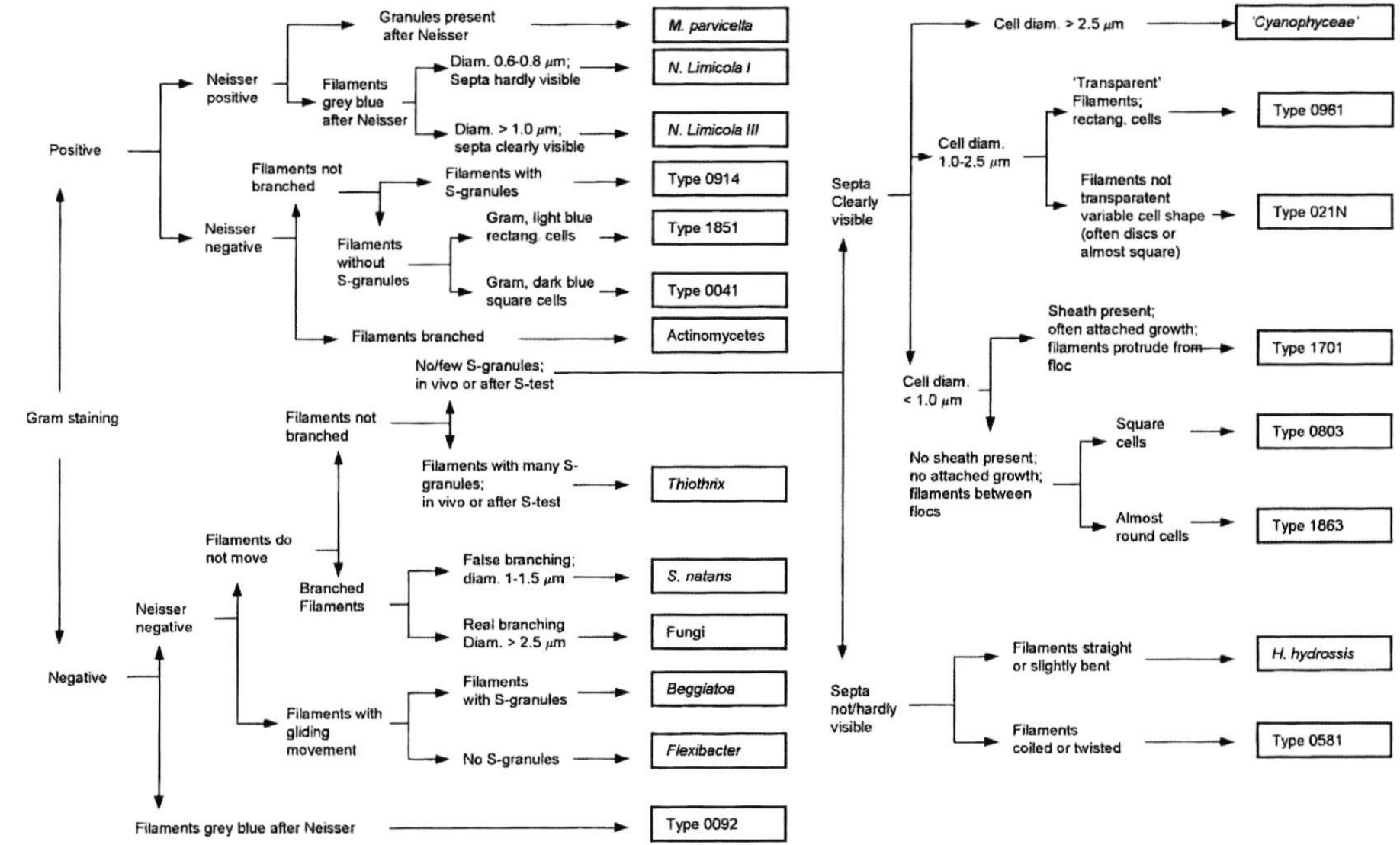
	Phase contrast microscopy															Bright-field Microscopy			
			Filament shape						diameter cells					S-granules in cells		Gram stain		Neisser stain	
	Branching	Motion	straight or somewhat bent	Coiled or twisted	Filament length < 200 µm	Attached growth	Sheath present	Septa clearly visible	< 1.0 µm	1.0-2.5 µm	> 2.5 µm	Cell shape square or rectangular	Cell shape round, disc or rod	In vivo	After Sulphur test	Positive	Negative	Positive	Negative
Actinomycetes	+			+	+				+			∞	∞			+		±	+
Beggiatoa		+	+		±				+	+	+	∞	∞	+			+		+
Cyanophyceae			+				±	+			+	+	+			+	+		+
H.hydrossis			+		+		+		+			∞	∞				+		+
Flexibacter		+	+		+			±	+			∞	∞				+		+
M.parvicella				+	+				+			∞	∞			+		+[6]	
N.limicola I				+	±			±	+				+			+		+[7]	
N.limicola III				+	±			+	+	+			+			+	9)	+[7]	9)
Fungi	+		+					+		+	+	+				4)	4)		+
S. natans	+[1]		+			±	+	+		+			+				+		+
Thiotrix	2)		+		±		±	+[3]	+	+		+[3]	+[3]	±	+		+		+
Type 0041			+		±	+	+	+	+	+		+				+			+
Type 0092			+		+				+			∞	∞				+	+[7]	
Type 021N			+		±			+	±	+		+	+		±[8]		+		+
Type 0581				+	+				+			∞	∞				+		+
Type 0803			+		+			+	+			+				±	+		+
Type 0914			+		+			+[3]	+			+[3]		+		+			+
Type 0961			+					+		+		+					+		+
Type 1701			+		+	+	+	+	+				+				+		+
Type 1851			+		±	+	+		+			∞	∞					+[5]	+
Type 1863				+	+			+	+				+				+		+

± = sometimes; ∞ = cell shape can not be discerned with phase contrast ;1) False branching ; 2) Sometimes rosettes; 3) Cells can be discerned after S-globules are dissolved ; 4) Fungi can not be stained with this method ; 5) light blue ; 6) granules ; 7) filaments stain grey blue ; 8) small granules ; 9) Gram and Neisser negative species exist too.

Identification key 1 of filamentous organisms in activated sludge

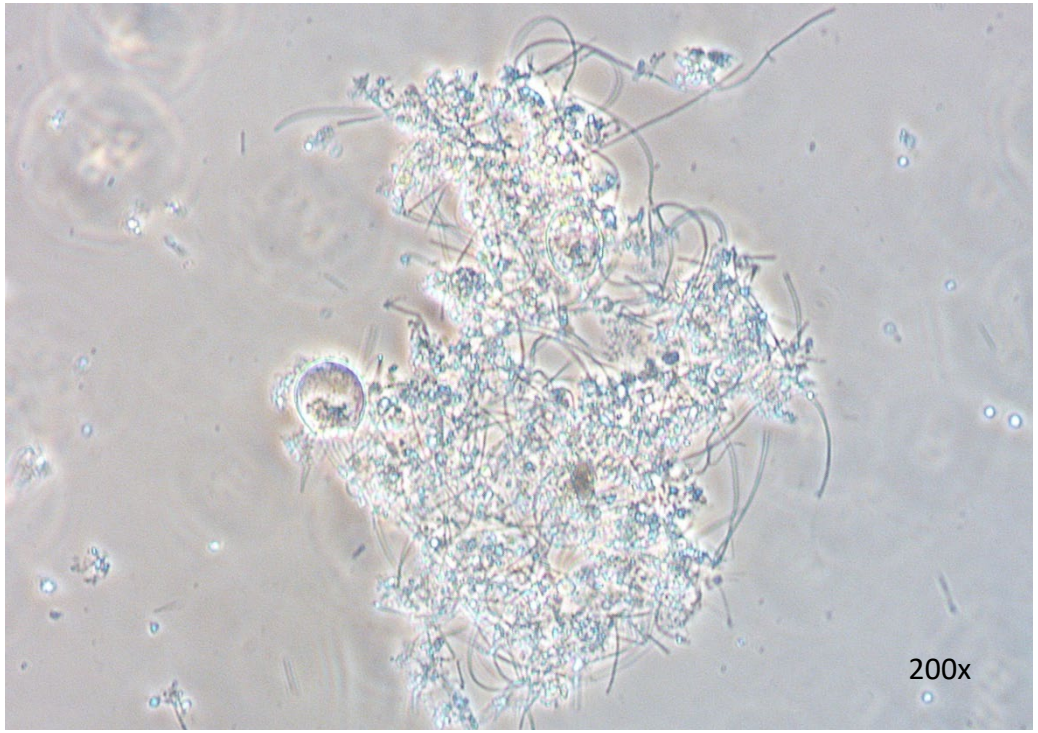


Identification key 2 of filamentous organisms in activated sludge



Case Study

- A local industrial plant's activated sludge had issues with bulking and settling
- The sludge also contained a lot of filaments
- The 11 filamentous characteristics were examined to identify the most prominent filament in the sludge



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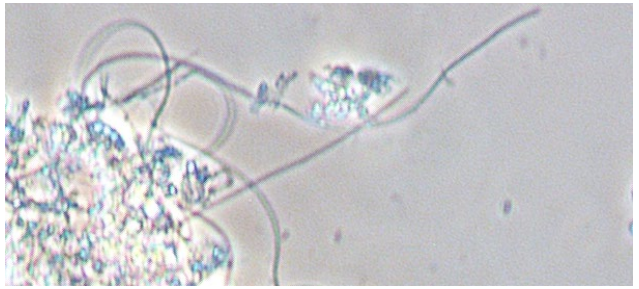
Identification 1 - Mobility

- Some filaments have a “gliding movement”, resembling how a worm moves itself
- Filamentous bacteria *Beggiatoa* and *Flexibacter* are known to do this

Beggiatoa leptomitoformis

https://www.microbiologyresearch.org/docserver/fulltext/ijsem/67/2/197_ijsem001584.pdf?expires=1618867469&id=id&accname=guest&checksum=9ADA4A3E92CFD4A2CC7EC94A863AA794

- Case Study:
  - *Did the filaments move?*
    - No



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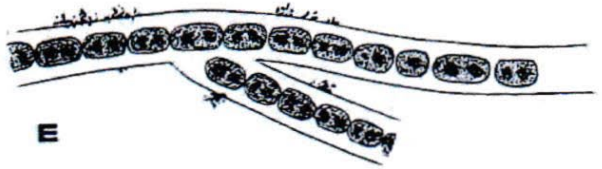
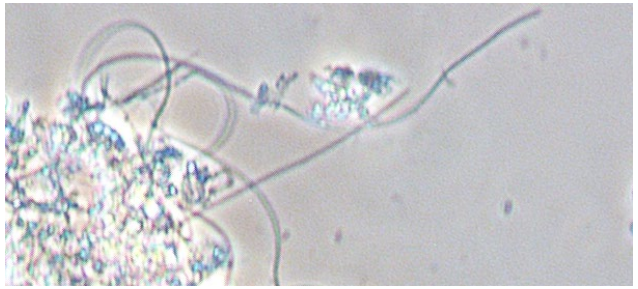
Identification 2 – Branching

- Real branching: the side shoot sprouts from the main branch and grows in 2 directions
  - Actinomycetes and fungi

- False branching: a sheathed filament has free-living cells (swarming cells) that attach to the sheath and develop into side shoots through cell division
  - *Sphaerotilus natans* and Type 1701



- Case Study:
  - *Did the filaments branch?*
    - No



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Identification 3 – Filament Shape

- Three groups of shape:
  - Straight
  - Bowed/bent
  - Twisted/coiled
- Straight filaments can still be somewhat bent, especially if they are long

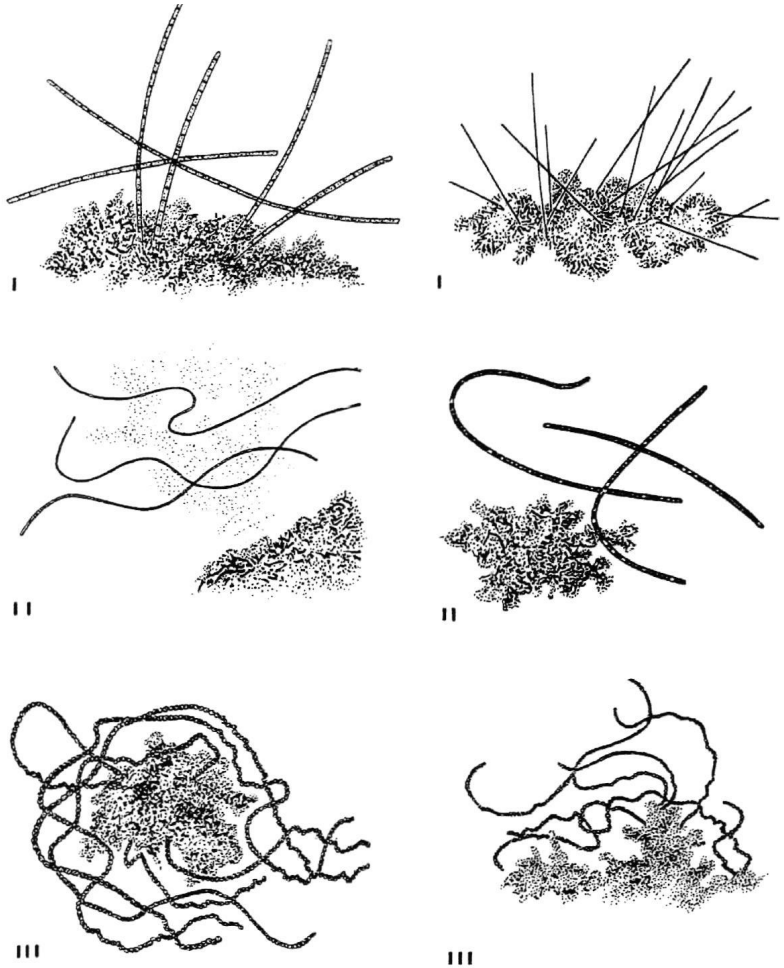
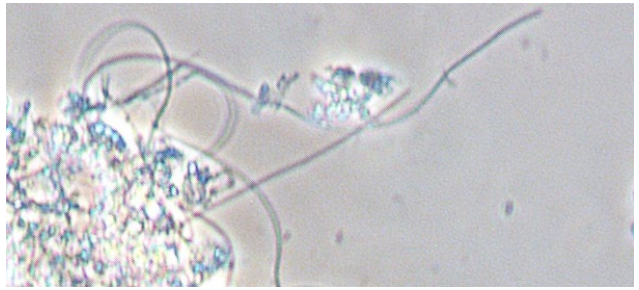


Figure 40 Shape of the filaments
I: straight filaments
II: bent filaments
III: twisted filaments.

- Case Study:
  - *What shape are the filaments?*
    - Straight/bent



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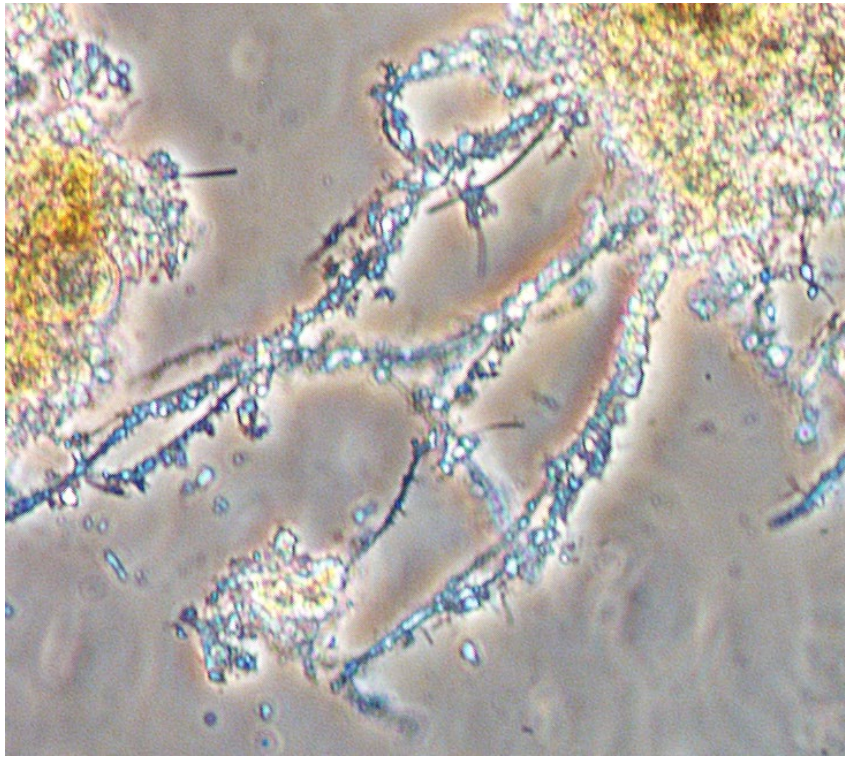
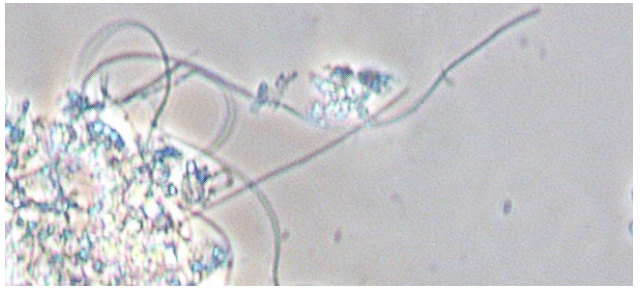
Identification 4 – Attached Growth

- Attached growth – Cells or particles of floc attached to the filament's sheath



- Case Study:
  - *Is there attached growth?*
    - No



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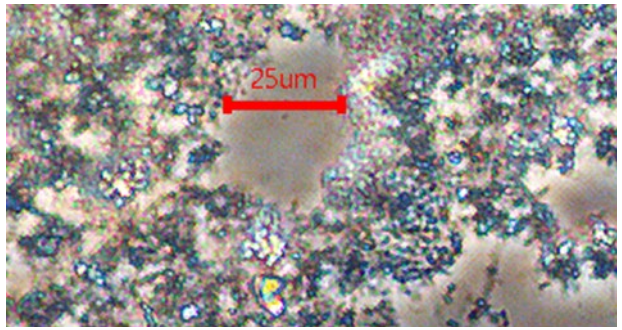
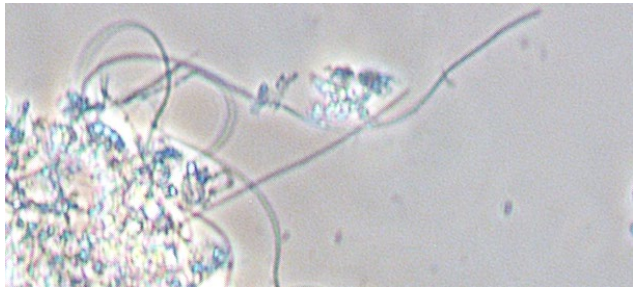
Identification 5 – Filament diameter

- To measure, must have:
  - Micrometer in one of the oculars
  - Microscope camera connected to a computer program
    - AmScope



- Three groups:
  - Diameter < 1 µm
  - Diameter 1 – 2.5 µm
  - Diameter > 2.5 µm
- For some filaments, diameter can decrease towards the tip of the filament
  - Type 021N and *Thiothrix*

- Case Study:
  - *What is the diameter?*
    - 1 – 2.5 µm



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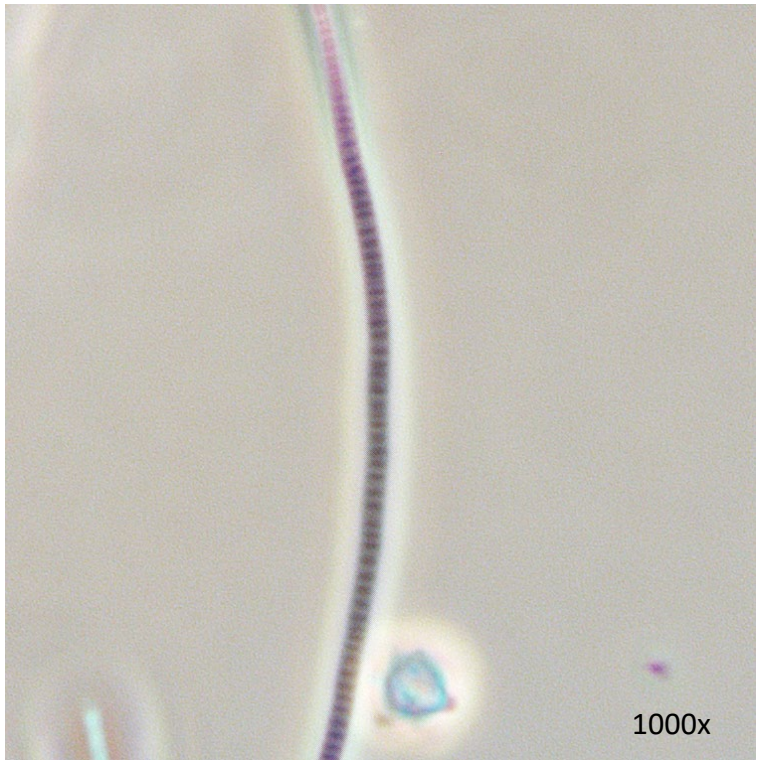
Identification 6 – Septa or Transverse Walls

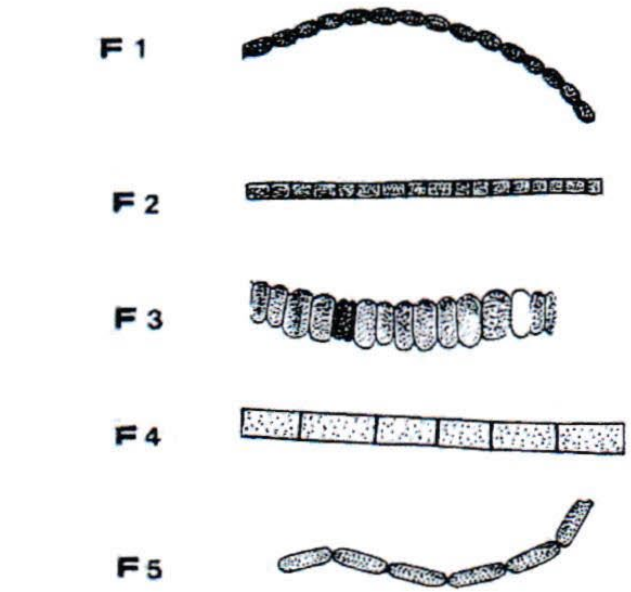
- Transverse walls or septa
  - Walls between the consecutive cells of the filament
- Two distinctions:
  - Clearly visible
  - Poorly visible/invisible

- Case Study:
  - *Are septa visible?*
    - Yes

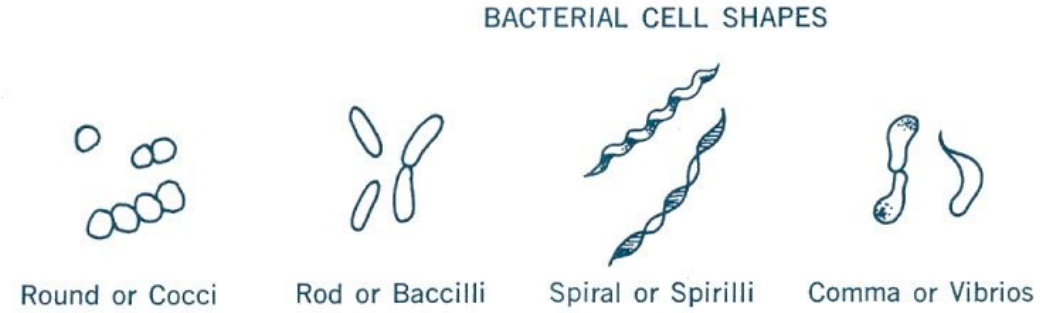


Identification 7 – Cell Shape

- Cell shape distinctions:
  - Spherical or coccus
    - Approximately round
  - Baccilus
    - Rod shaped
    - Square
    - Disc shaped
    - Rectangular
  - Spiral shaped
    - The length of the cells are much greater than cell diameter
      - Spirils and Spirochaetes
  - Vibrio shaped
    - Cells are somewhat bowed



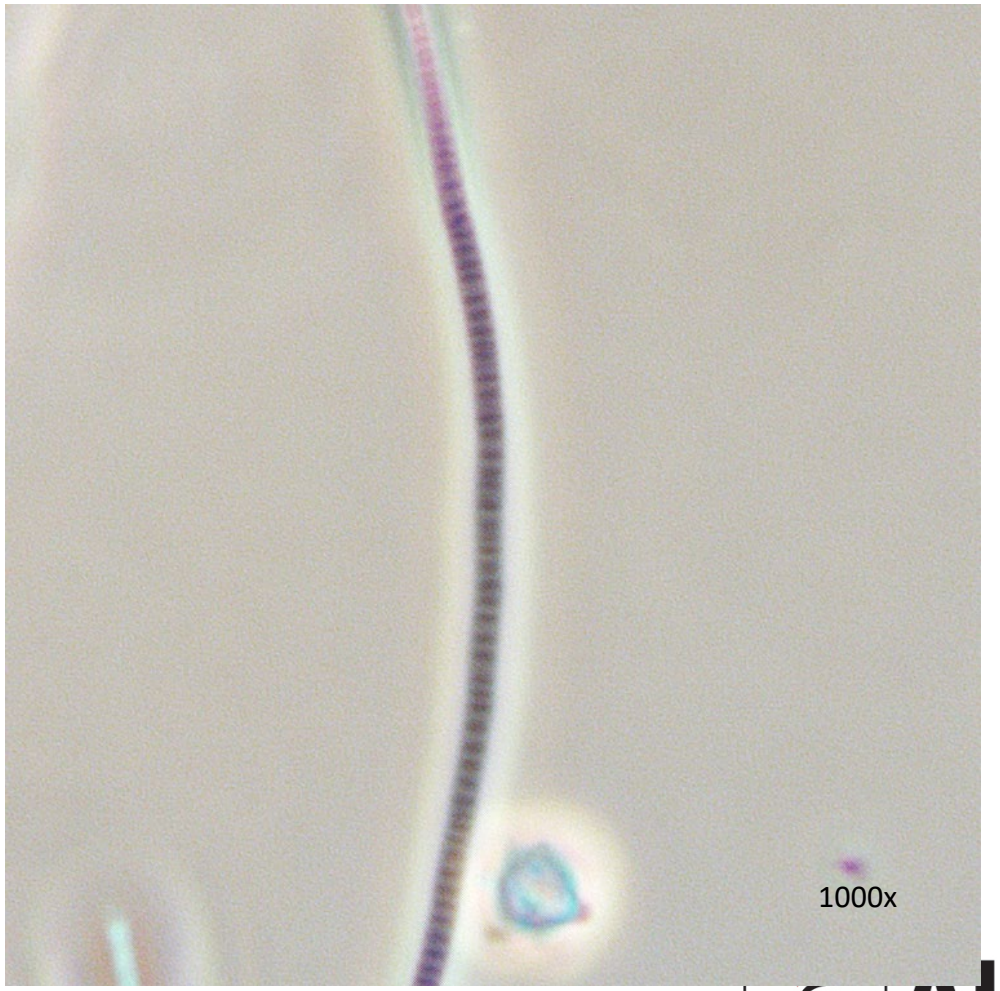
F: Cell shapes; (1) almost round, (2) square, (3) disc shaped, (4) rectangular, (5) rod shaped.



https://emilybiologyinteractivenotebook.wordpress.com/2012/08/05/entry-8-cell-tissue-organ-system-organism/

Identification 7 – Cell Shape

- Case Study:
  - *What is the cell shape?*
    - Disc shaped/square



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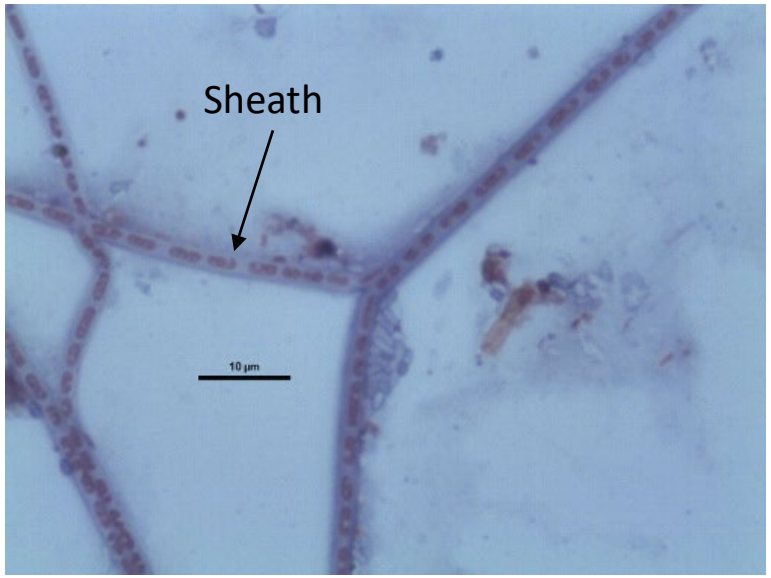
Identification 8 - Sheath

- Some filamentous species are surrounded by a transparent cover (sheath)
  - Difficult to see with a light microscope
    - Can sometime tell after gram staining
  - A lot of attached growth can indicate sheath presence



https://teamaquafix.com/sphaerotilus-natans-s-natans-treatment/

- Case Study:
  - *Is sheath present?*
    - Unable to determine

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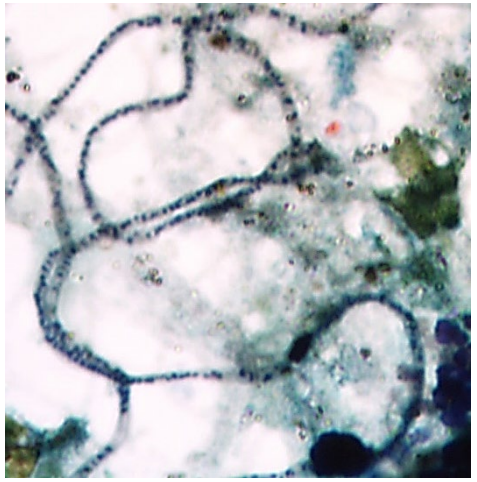
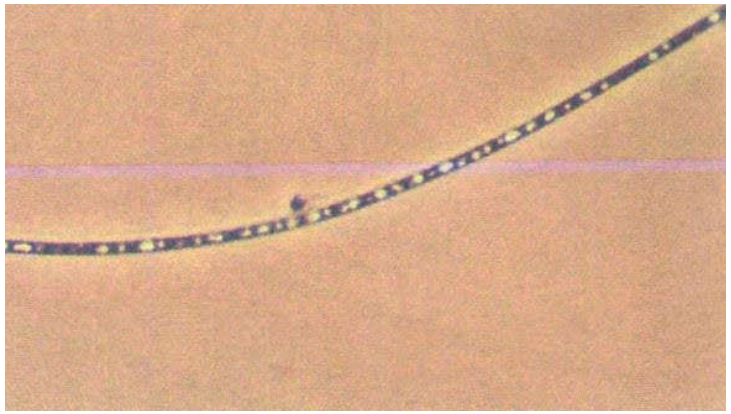
Identification 9 - Granules

- Granules – globules of storage material in the cells
  - Polyphosphate granules
    - Visible via Neisser staining
  - Sulfur granules
  - Other storage materials





Jenkens, D., Richard, M.G. and Daigger, G.T. (2004). *Manual on the Causes and Control of Activated Sludge Bulking, Foaming, and Other Solids Separation Problems.* 3rd ed.. Boca Raton, FL: CRC Press LLC.

- Case Study:
  - *Granules present?*
    - No

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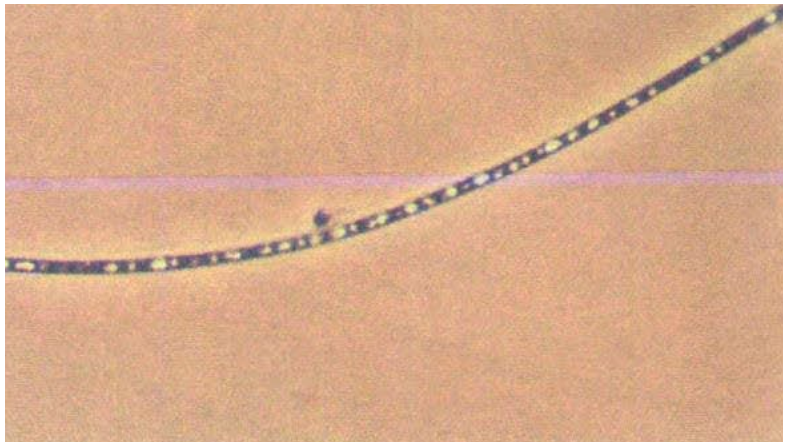
Sulfur Storage Test

- Make solution:
  - 0.1 g Sodium Sulfide ($Na_2S \cdot 9H_2O$)
  - 100 mL Distilled water
- Procedure:
  - Mix one drop of sludge with one drop of sodium sulfide solution on a slide
  - Let stand to open air for 10 – 20 minutes
  - Place coverslip on slide
  - Examine under 1000x (oil immersion) with phase contrast
  - If positive, granules will be highly refractive and yellow-colored



Jenkens, D., Richard, M.G. and Daigger, G.T. (2004). *Manual on the Causes and Control of Activated Sludge Bulking, Foaming, and Other Solids Separation Problems.* 3rd ed.. Boca Raton, FL: CRC Press LLC.

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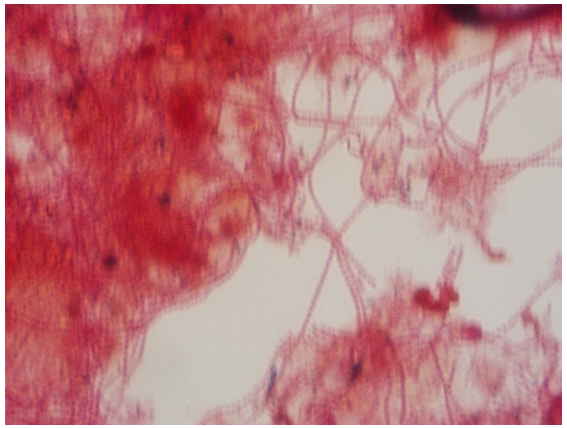
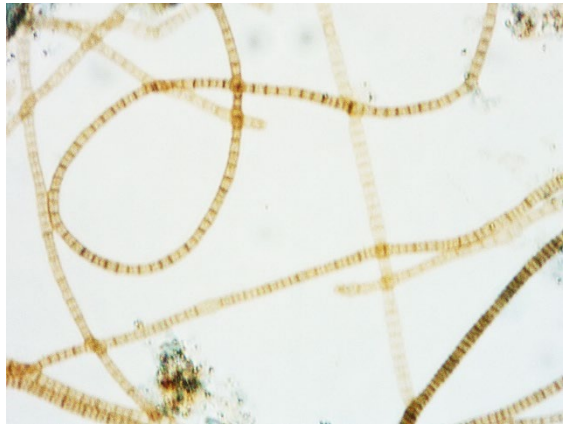
Identification 10 & 11 – Gram & Neisser Staining Results

- Case Study:
  - *Gram stain?*
    - Negative
  - *Neisser stain?*
    - Negative





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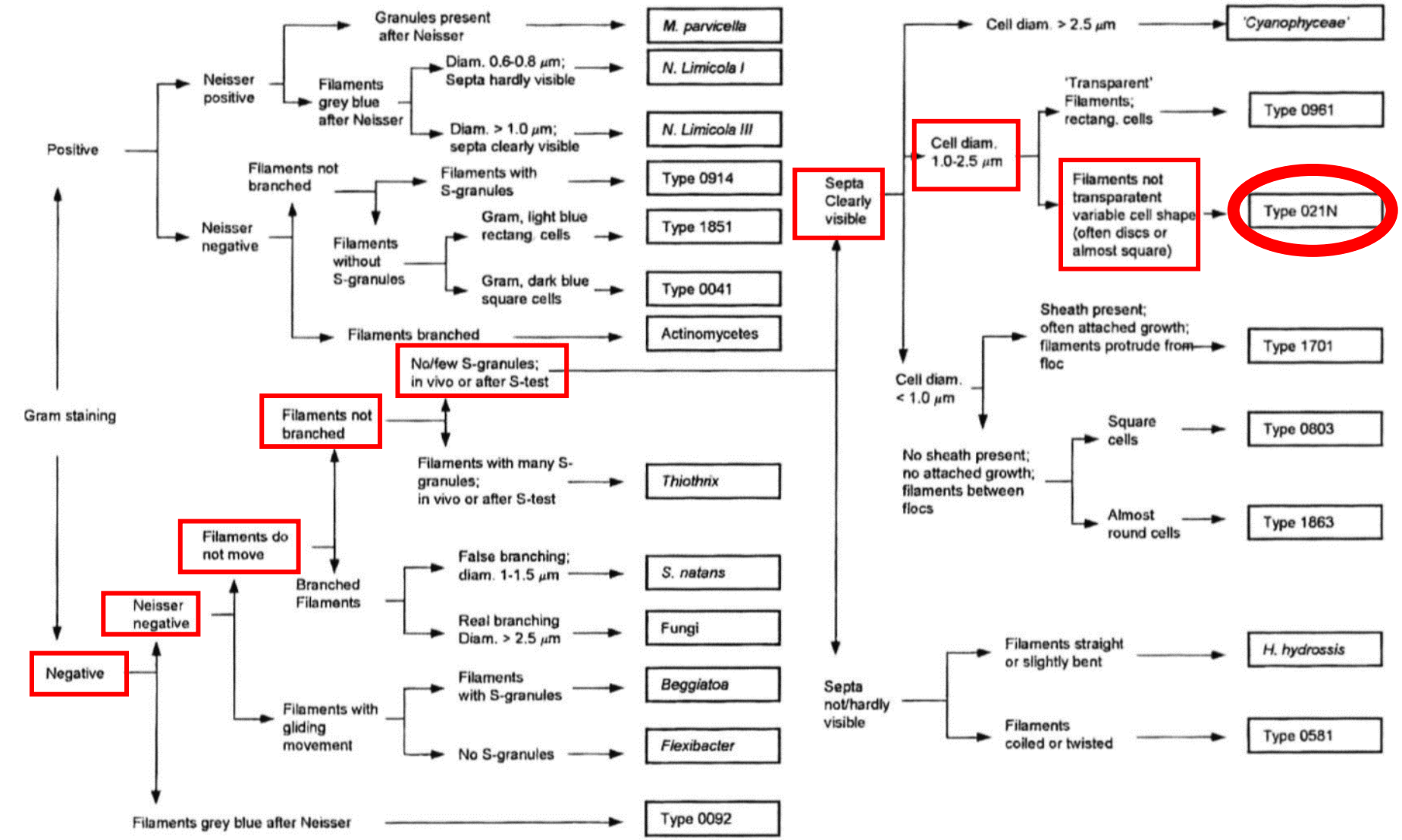
Identification key 2 of filamentous organisms in activated sludge



Identification: Type 021N

- The sludge predominately contained Type 021N filaments
  - Related to *Thiothrix*
  - Common in activated sludge
  - Fast growing, aerobic species
  - Growth: stimulated by unbalanced influent nutrient composition (lacking N, P, etc.) and low oxygen concentrations in aeration tank
  - Causes bulking
  - Control strategies:
    - Good housekeeping
    - Remove deficiencies: O2 > 2 mg/L and BOD:N:P = 100:5:1
    - Do a two step configuration:
      - Aerobic/aerobic or anaerobic/aerobic
      - Removes easily degradable influent fraction before it enters aeration tank
    - Control symptoms
      - Apply physical and chemical methods used to destroy filaments or improve settling velocity of floc by increasing weight

https://www.asissludge.com/pdf/Thiothrix21n.pdf

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Activated Sludge Evaluation

Eikelboom, D.H. (2000). *Process Control of Activated Sludge Plants by Microscopic Investigation.* London, UK: IWA Publishing.

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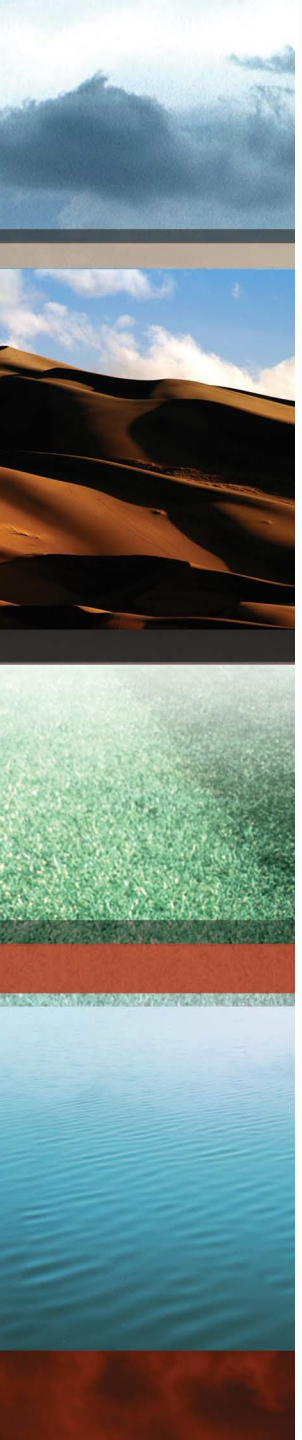

Activated Sludge Evaluation

- To evaluate the condition of a plant's activated sludge, factors other than filament identification are considered
  - Floc Shape
  - Floc Structure
  - Floc Strength
  - Floc Size
  - Floc Diversity
  - Free-living cells
  - Spirochaetes/Spirils
  - Filament Index
  - Protozoa and Metazoa

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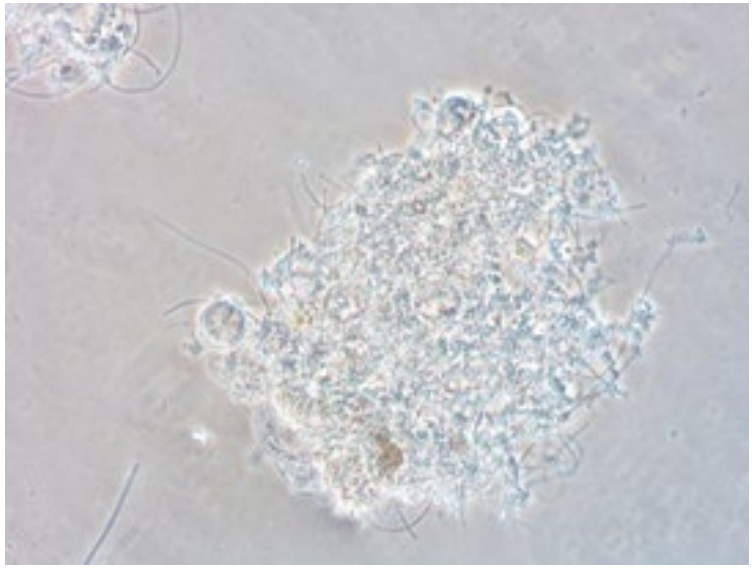
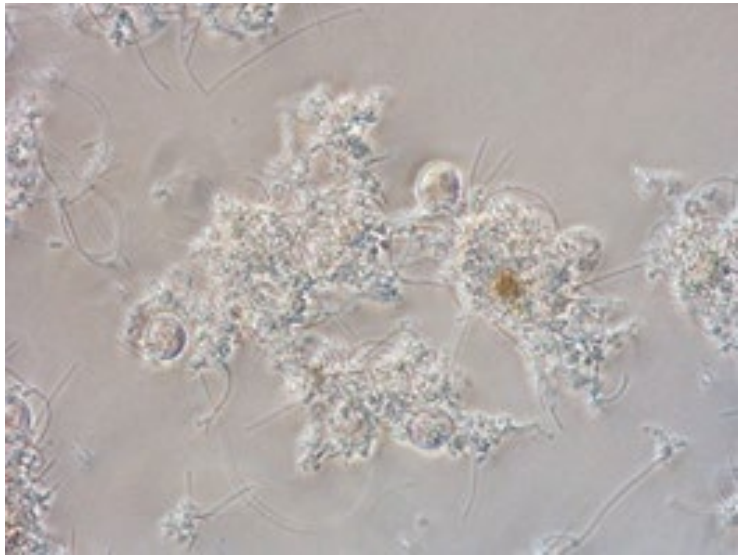
Floc Shape

- Floc shape is characterized as:
  - Round



- Irregularly shaped
  - Can reduce settling velocity



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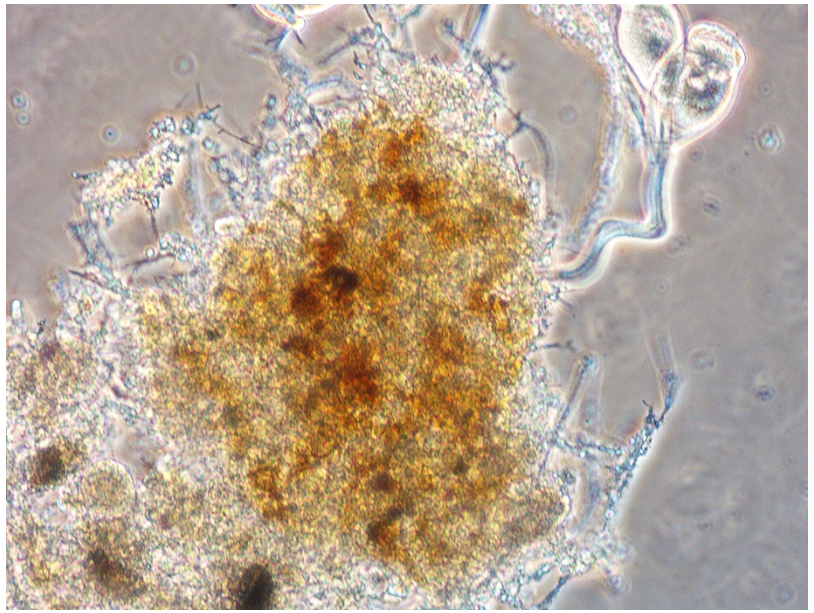
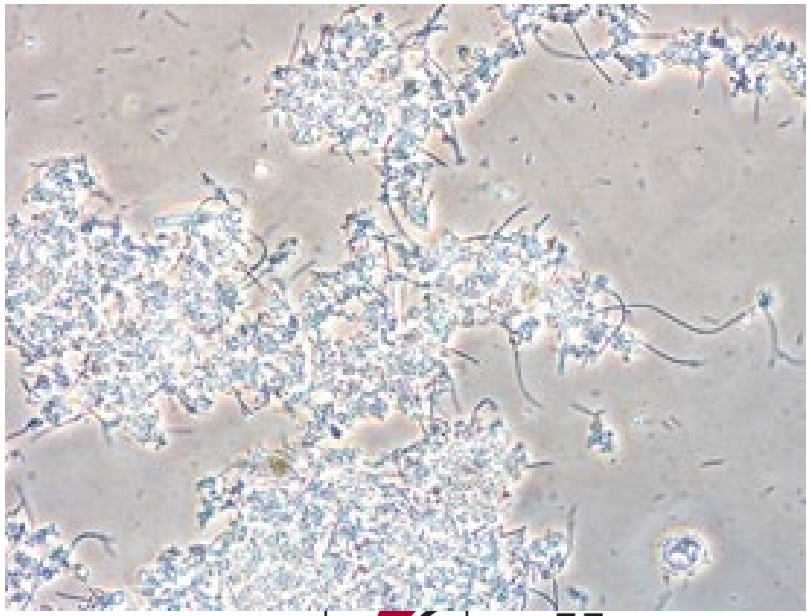
Floc Structure

- Compact flocs
  - Bacteria are stacked close to one another
  - Floc is mostly brown
  - Settles faster



- Open flocs
  - Water can flow through the floc particles
  - Slow setting



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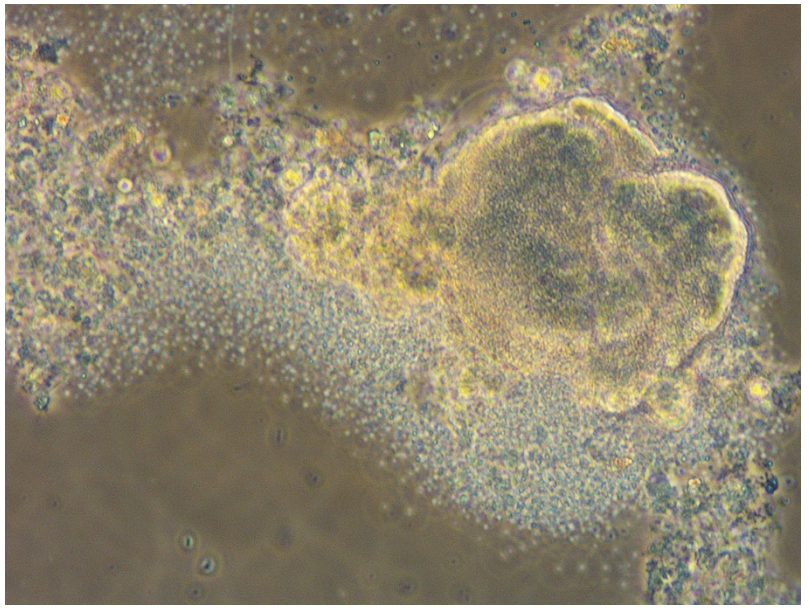
Floc Strength

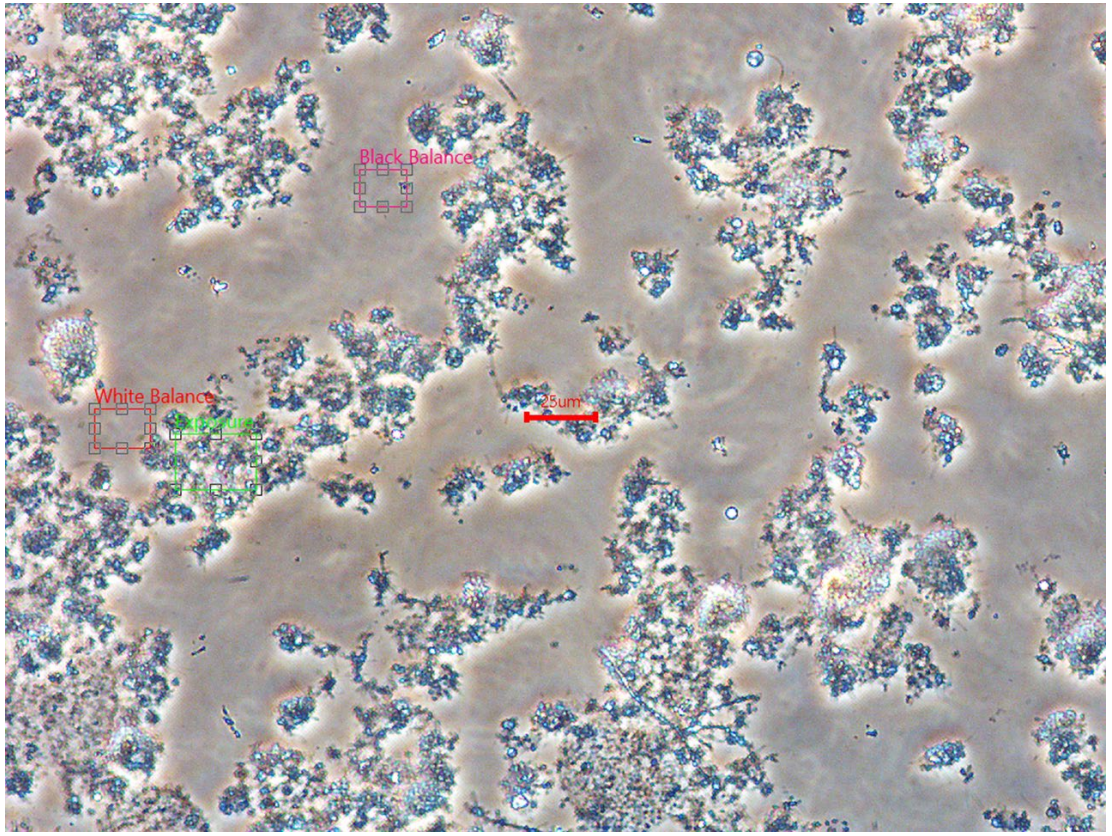
- Firm
  - The floc and surrounding liquid are separated
  - Low loaded influent

- Weak
  - Little definition from surrounding liquid
  - Free cells at edges of floc
  - High loaded influent
  - Flocs can be easily damaged



Floc Size

- Three size classes:
  - Small flocs
    - Diameter < 25 µm
  - Medium flocs
    - Diameter 25-250 µm
  - Large flocs
    - Diameter > 250 µm
- Large flocs settle more quickly
- A high percentage of small flocs (>25%) can result in sludge being discharged with the effluent
  - Can be caused by:
    - Highly turbulent diffusion
    - Low sludge load
    - Aluminum salts
    - Toxicity



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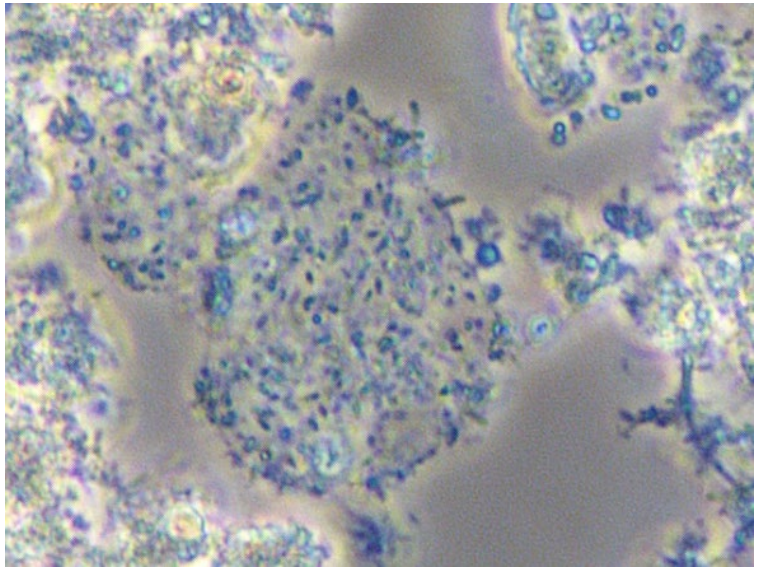
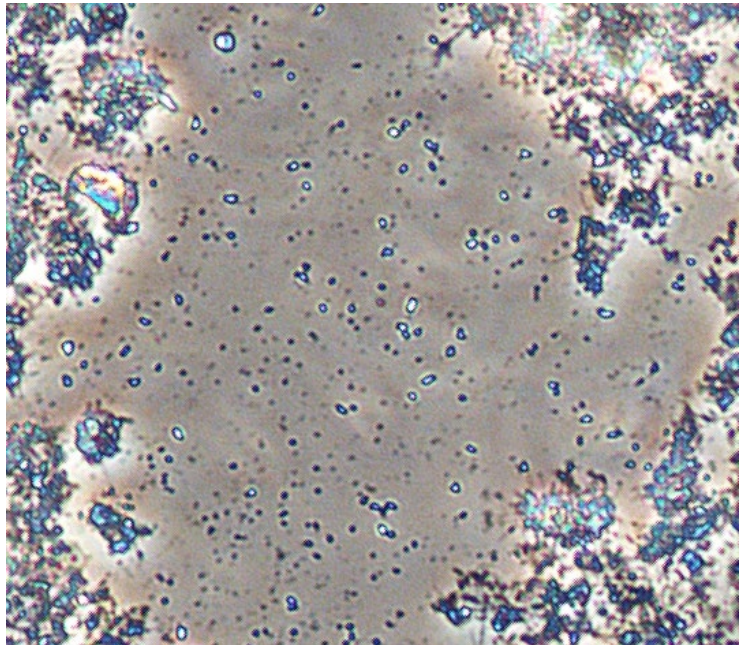
Floc Diversity

- Low diversity of microorganisms
  - Can make a plant more vulnerable in the event of a plant upset
  - Occurs in high loaded treatment plants fed with an unbalanced influent
- Higher diversity allows other microorganisms to perform functions primarily performed by bacteria that may have died during a plant upset
- Monocolonies
  - Conglomerates of one type of bacteria held together by a slime matrix
  - Some monocolonies are indicative of nutrient shortages and high sludge loads
    - Zoogloea colonies
    - Monocolonies with a thick slime matrix



Free-Living Cells

- Cells that are not bound to the floc
- Do not settle in the final clarifier
  - Excess free-living cells can affect final effluent quality
- Indicative of high loading levels or low oxygen levels



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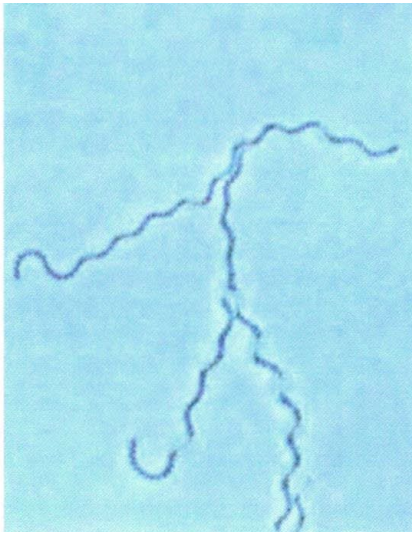
Spirochaetes/Spirils

- Spirils
  - One coil with bowed cells
  - Corkscrew-like movements
- Spirochaetes
  - Two coils – one short and one long
  - “Wriggle” movements
- Both indicate lack of oxygen in the plant



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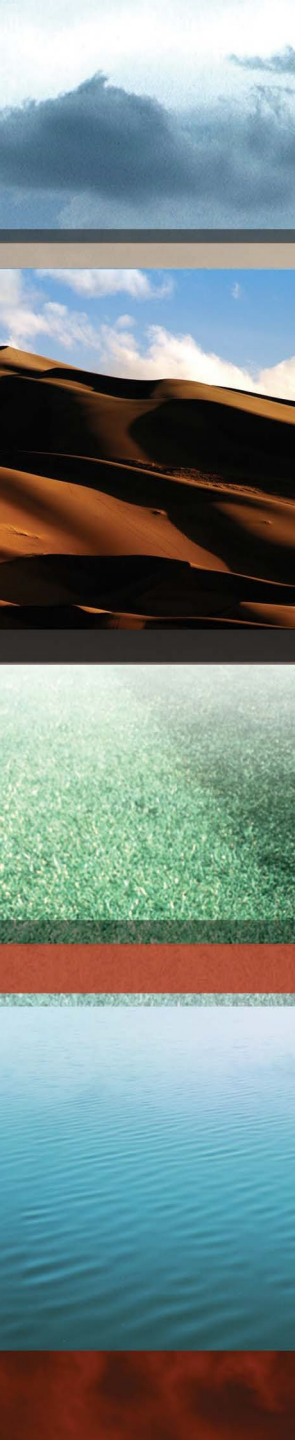

The Filament Index

- A measure of the filamentous organisms in activated sludge
- Scale of 0 – 5 (0 being absent and 5 being very prevalent)
- Compare the sludge to the series of reference photos
- Filament Index (FI) of 1 to 2:
  - Effect on settling velocity is slight
- FI of 3
  - Settling properties noticeably deteriorate
- Sludge bulking often occurs at higher FI

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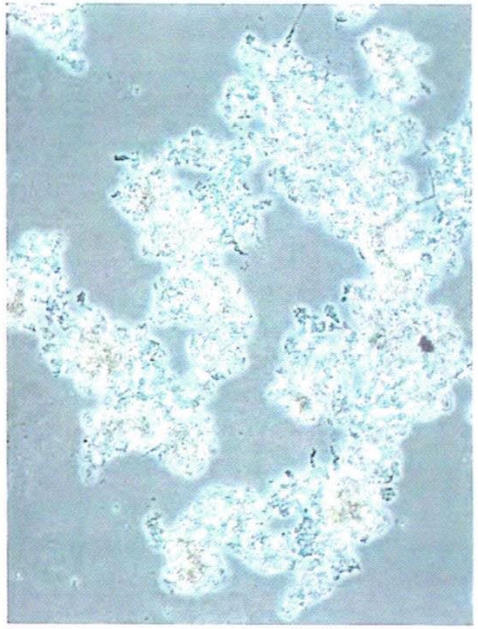


Figure 29 FI = 0 (150×).

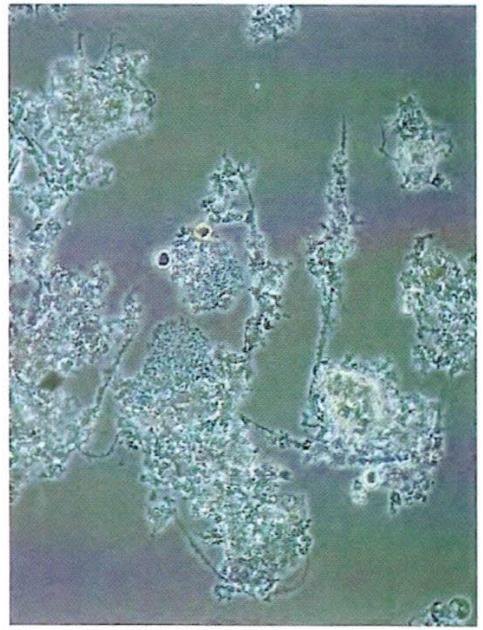


Figure 30 FI = 1 (150×).

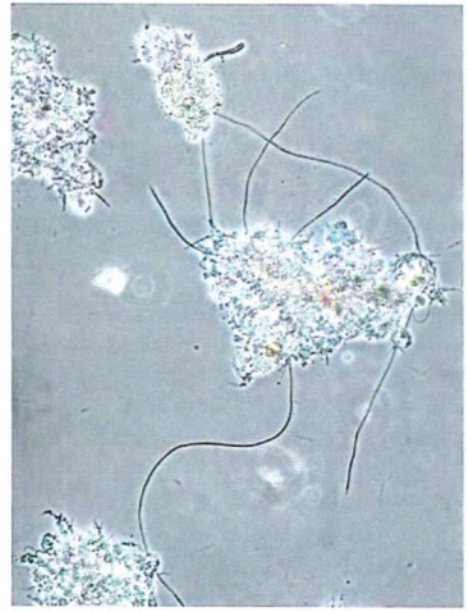


Figure 31 FI = 2; robust filaments (150×).

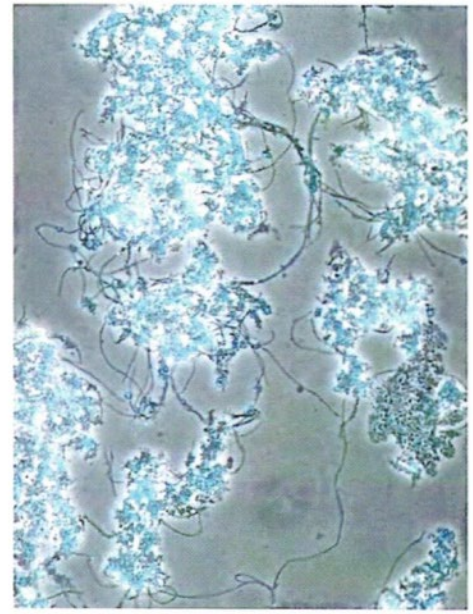


Figure 32 FI = 2; thin filaments (300×).

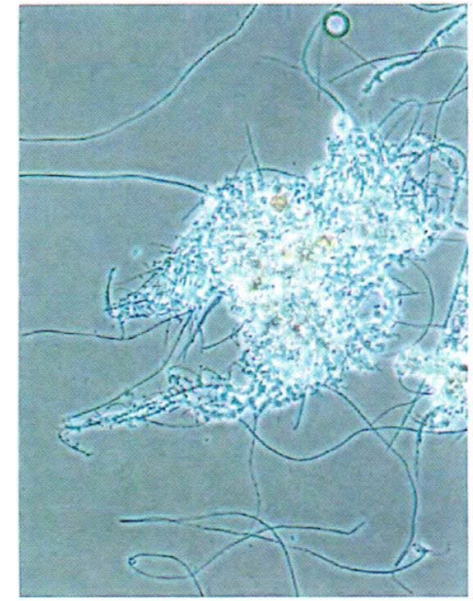


Figure 33 FI =3; robust filaments (150×).

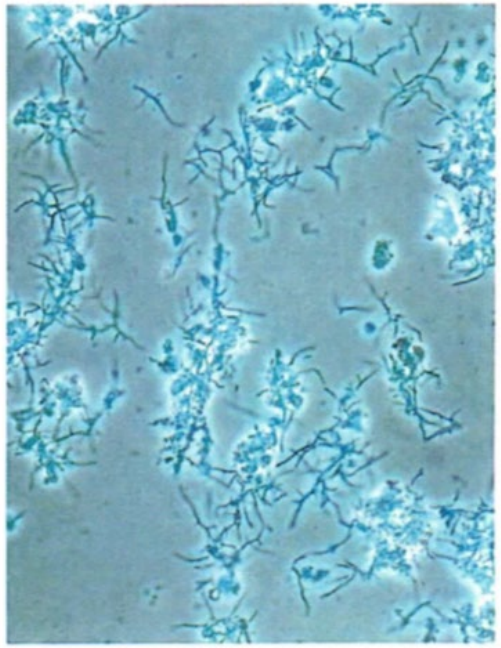


Figure 34 FI = 3; thin filaments (300×).

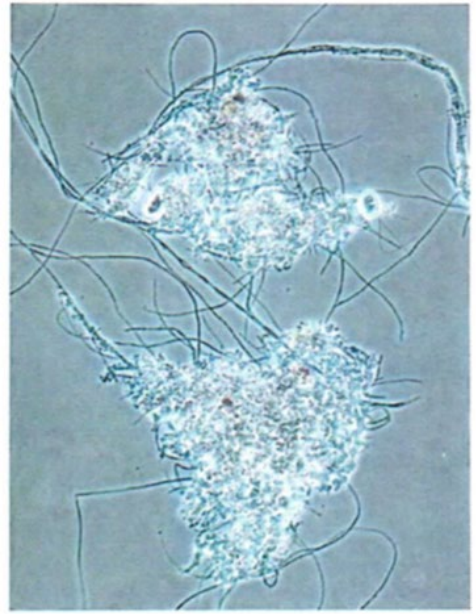


Figure 35 FI =4; robust filaments (150×).

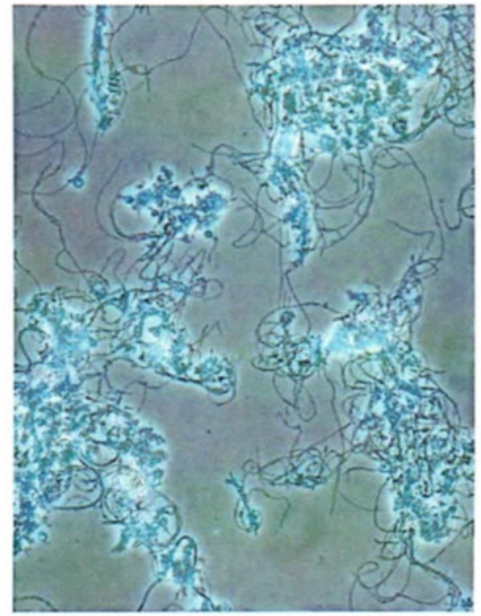


Figure 36 FI = 4; thin filaments (300×).

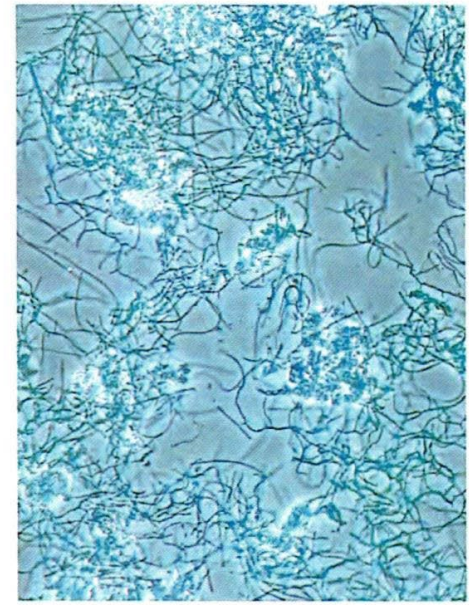


Figure 38 FI = 5; thin filaments (300×).

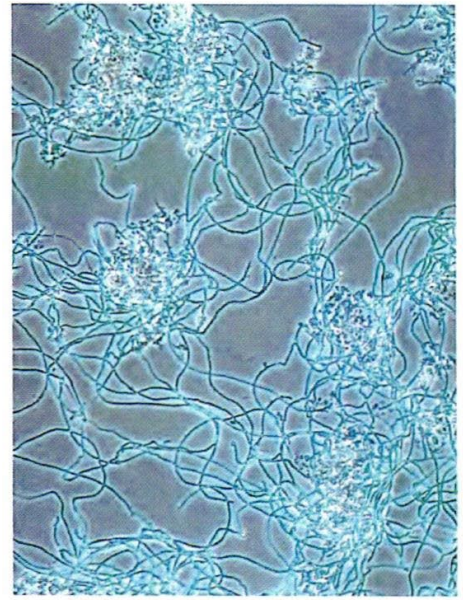
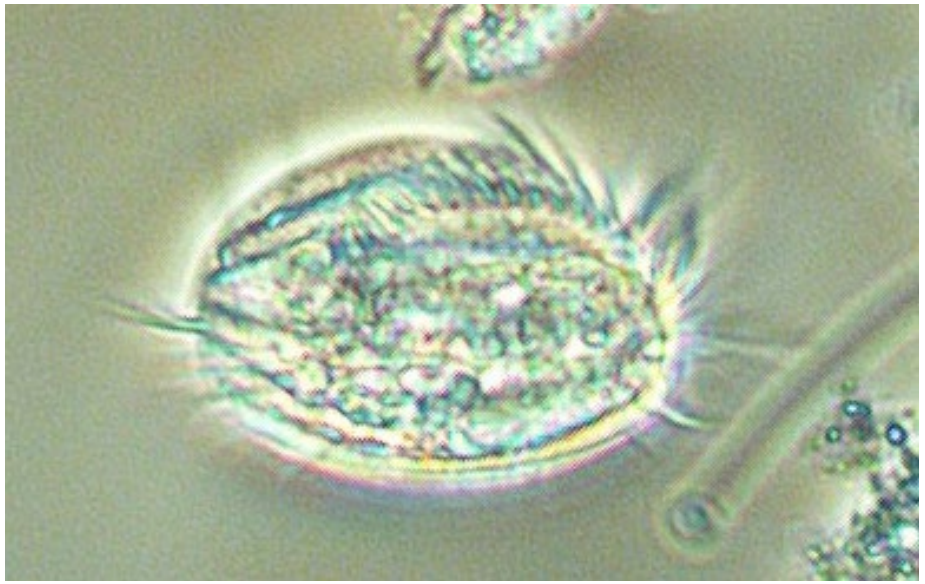


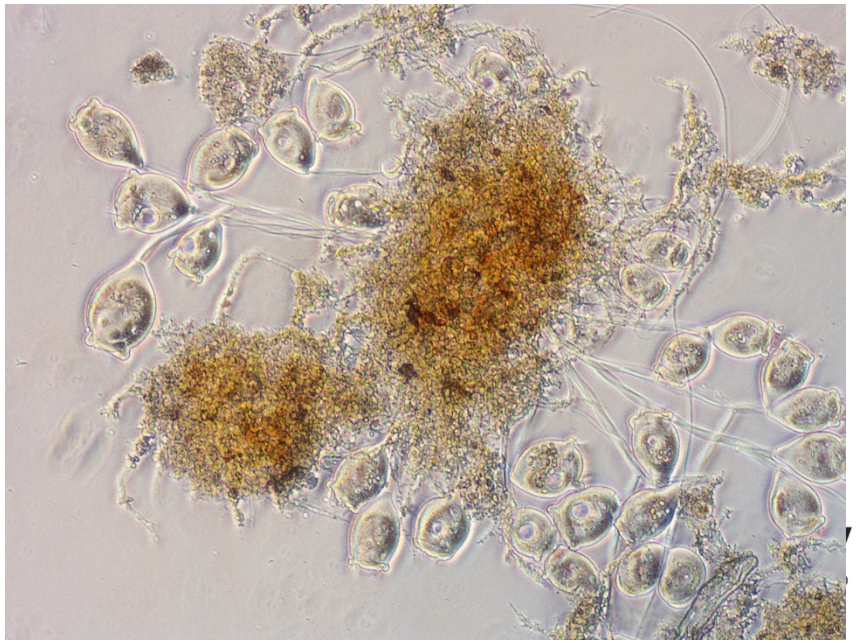
Figure 37 FI =5; robust filaments (150×).

Protozoans

- Free swimming ciliates
  - Move freely within the sludge
  - Have vibrating hairs (cilia)
  - Population size correlates to sludge load



- Stalked Ciliates
  - Positioned on a stem
  - Have vibrating hairs (cilia)
  - Population size correlates to sludge load



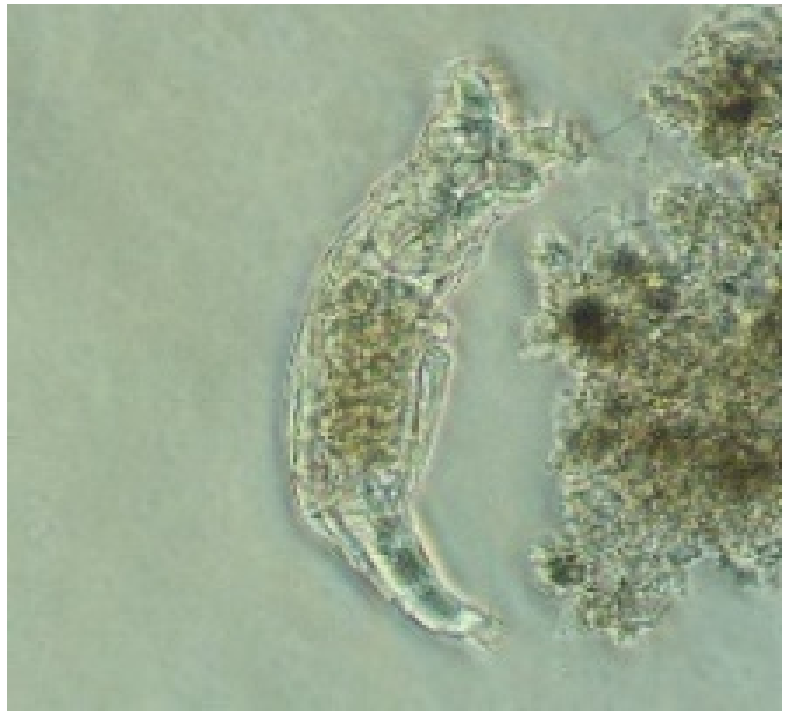
Protozoans

- Flagellates
  - Possess one or more flagella
  - Indicates high sludge loads
  - Rapid movements
  - Smaller than ciliates

- Amoeba
  - Constantly changing shape (flexible cell membrane)
  - Move very slowly with pseudopodia
  - Indicates high sludge loads and oxygen shortages

Metazoans

- Rotifers
  - Elongated with branched tail
    - Uses it to move
    - Attaches itself to the substrate, stretches its body, then releases its tail
  - Have cilia on their head
    - Creates a current to bring particles to their mouth
  - Their “jaws” allow them to break particles filtered from the water
  - Indicates low sludge loads



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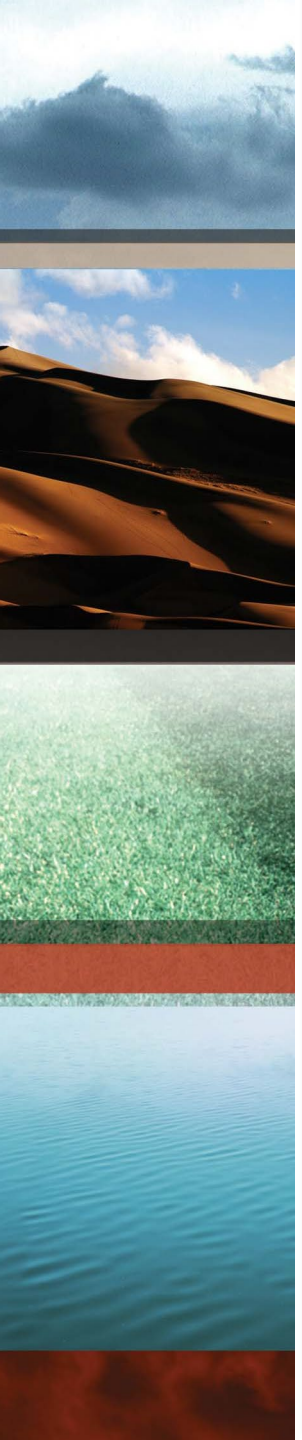
Metazoans

- Nematodes
  - Elongated, cylindrical, and flexible bodies
  - Regularly observed in low sludge loads

- Worms
  - Largest organisms that can be observed during microscopic exam
  - Indicates old sludge

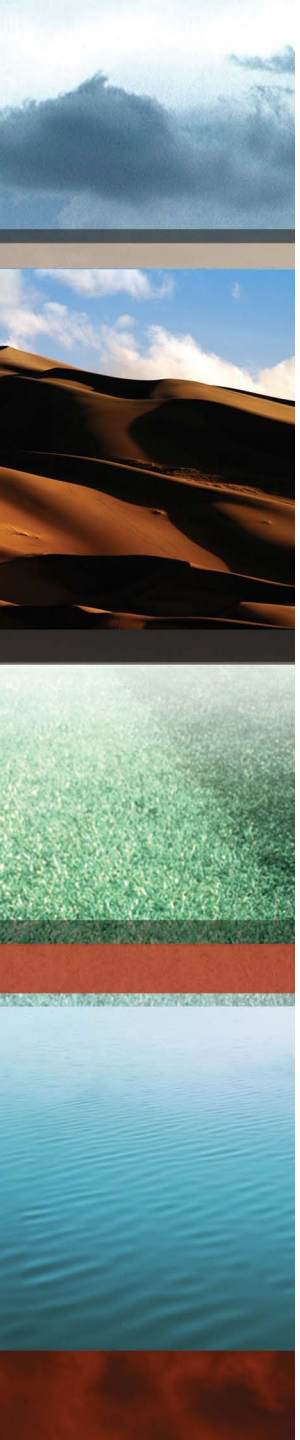
https://www.environmentalleverage.com/Flash%20wastewater%20training/Worms/slides/

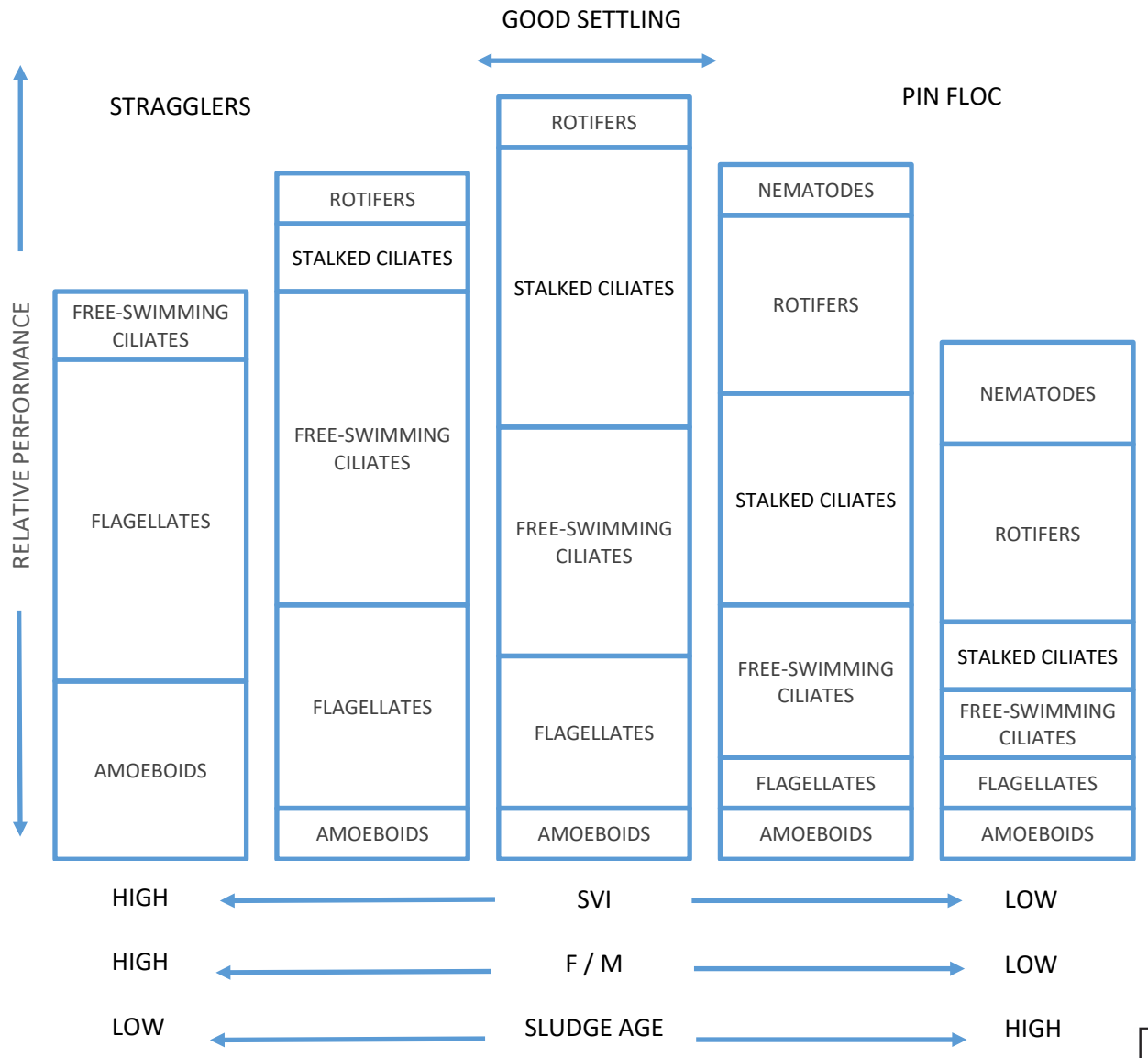


Sludge Quality Characterization

- First, rate the presence of the following on a scale of 0-3 (0 = absent, 3 = very prevalent)
  - Free-living cells
  - Spirils/spirochaetes
  - Ciliates
  - Flagellates
  - Amoebas
- Then, compare results of examination to the table
- If one criteria is moderate and the rest are good, the sludge is considered "good"
- If 2-3 criteria are moderate, then the sludge is considered "moderate"
- If most criteria are considered moderate or there is one criteria that is poor, then the sludge is considered "poor"

	Good	Moderate	Poor
Filament Index	< 3	3 – 4	4 – 5
Free-living cells	0 – 1	2 – 3	3
Spirils	0	1	≥ 2
Ciliates	> 1	< 1	0
Flagellates / Amoeba	0	1 – 2	≥ 3
% Flocs > 25 µm	> 80 to 90	> 50 to 70	≤ 50
Floc structure	Compact	Open	-
Floc strength	Robust	Weak	-
Floc shape	Rounded	Irregular	-





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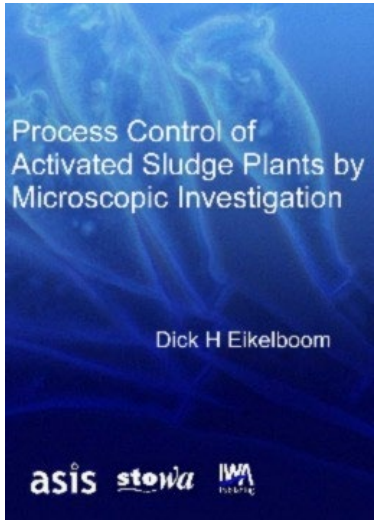
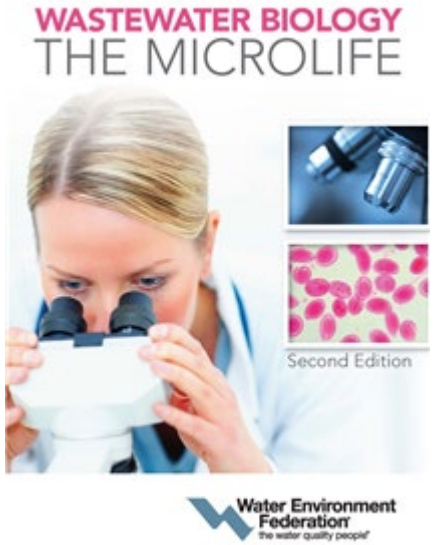
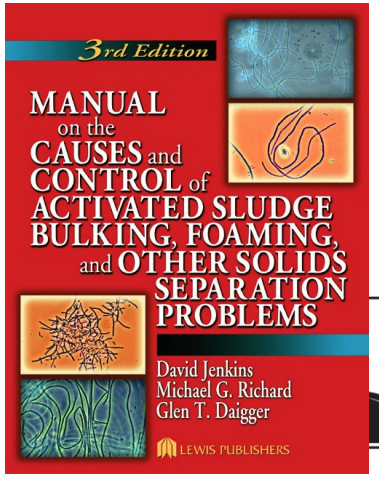
Resources

- The following resources aid in the microscopic examination process and also provide insight on operation controls and troubleshooting
  - Eikelboom, D.H. (2000). *Process Control of Activated Sludge Plants by Microscopic Investigation*. London, UK: IWA Publishing.
  - Water Environment Federation (2001). *Wastewater Biology: The Microlife*. Alexandria, VA: Water Environment Federation.
  - Jenkens, D., Richard, M.G. and Daigger, G.T. (2004). *Manual on the Causes and Control of Activated Sludge Bulking, Foaming, and Other Solids Separation Problems*. 3rd ed. Boca Raton, FL: CRC Press LLC.







Thank you!

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