

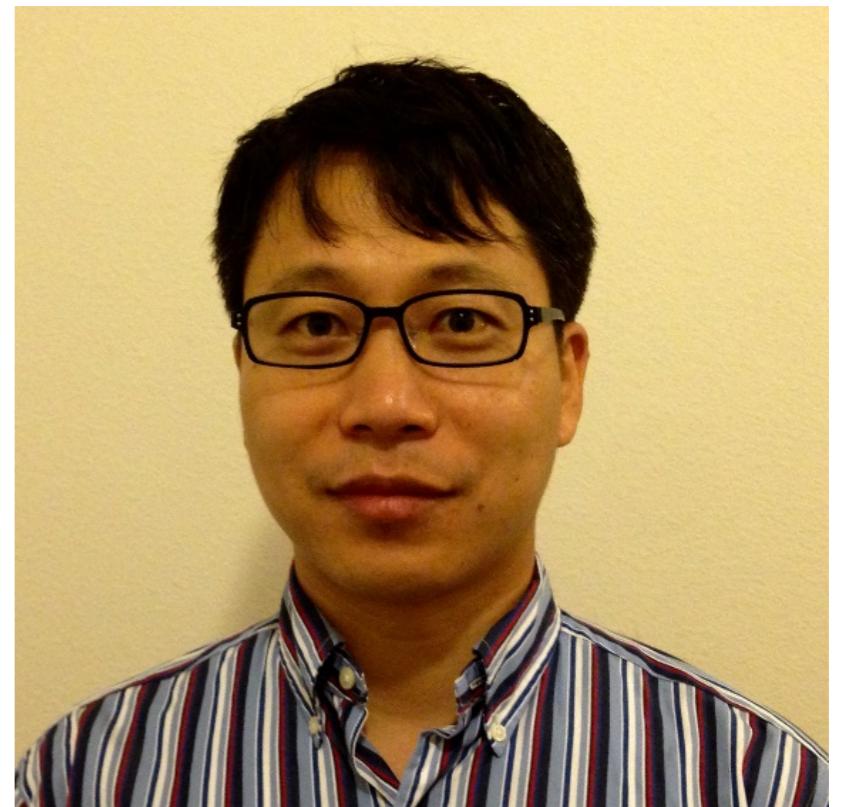
ESE 5001

Environmental Engineering Principles

Biological Principles

Introduction

- Lecturer:
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 - Office: E2-04-07
 - Telephone: 6516-2884
 - Email: ceebsw@nus.edu.sg



Lecture time

- ◆ Option 1

6:00 ~ 6:45 pm: Lecture

6:45 ~ 7:00 pm: 15 min break

7:00 ~ 7:45 pm: Lecture

7:45 ~ 8:00 pm: 15 min break

8:00 ~ 8:45 pm: Lecture

8:45 ~ 9:00 pm: 15 min break

- ◆ Option 2

6:00 ~ 7:00 pm : Lecture

7:00 ~ 7:20 pm : 20 min break

7:20 ~ 8:35 pm Lecture

Topics

1. General introduction of Microbiology
2. Molecular microbial techniques
3. Microbial community analysis
4. Antibiotics Resistance

Topic 1

General introduction of

Microbiology

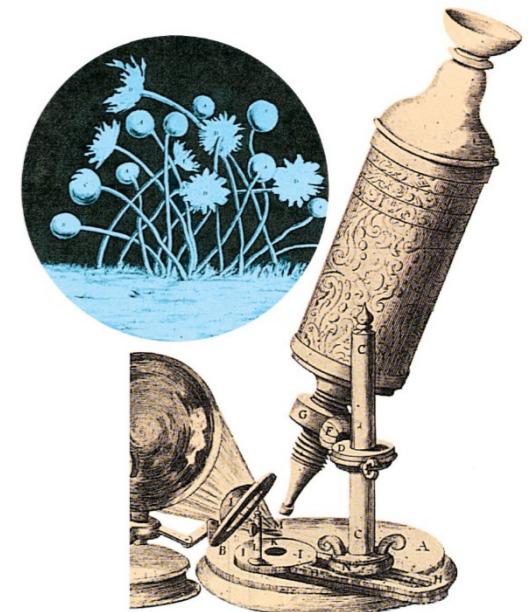
Outline

- What is Microbiology ?
- Why is microbiology important?
- Microbes
- The Impact of Microorganisms on Human
- Microbiology for Environmental Engineers

What is Microbiology

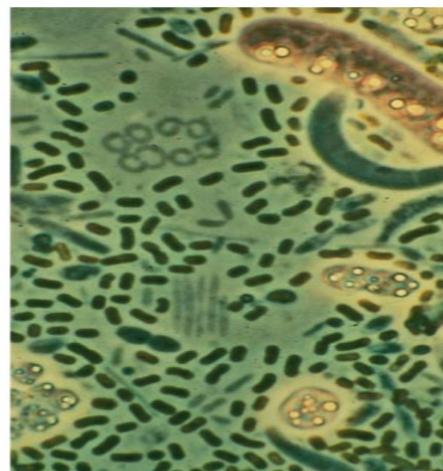
- Study of Microorganisms

- Microorganisms entities **Virus (NOT cells)**
 - Prokaryotes: Bacteria and Archaea
 - Eukaryotes
- Single cells, cell clusters, biofilms
- Free-living: not directly dependent on another organism for survival.
 - Distinct from plant/animal cells, which exist only as part of multi-cellular organism

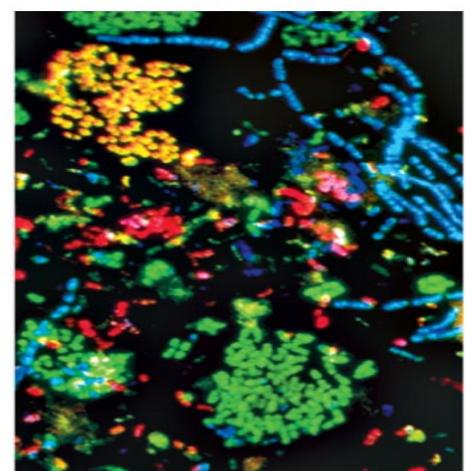


Why is it important?

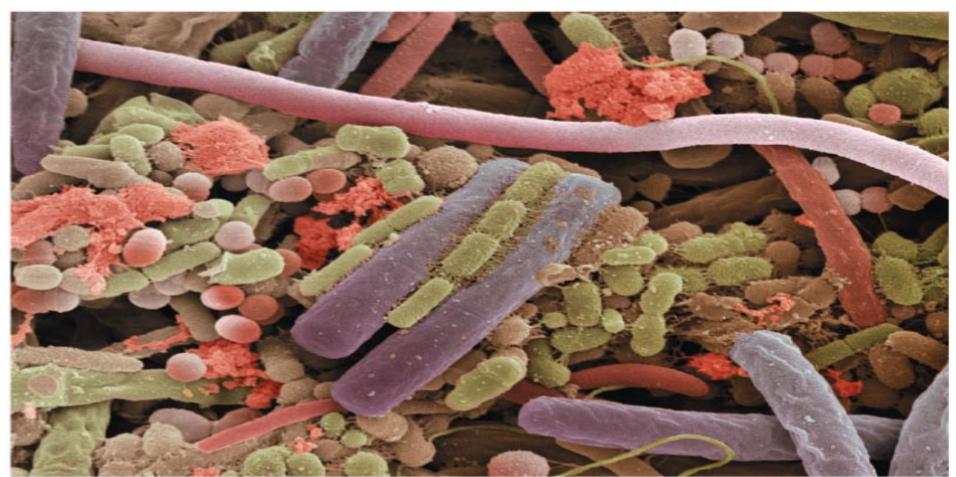
My personal journey of environmental engineering
and microbiology....



(a)

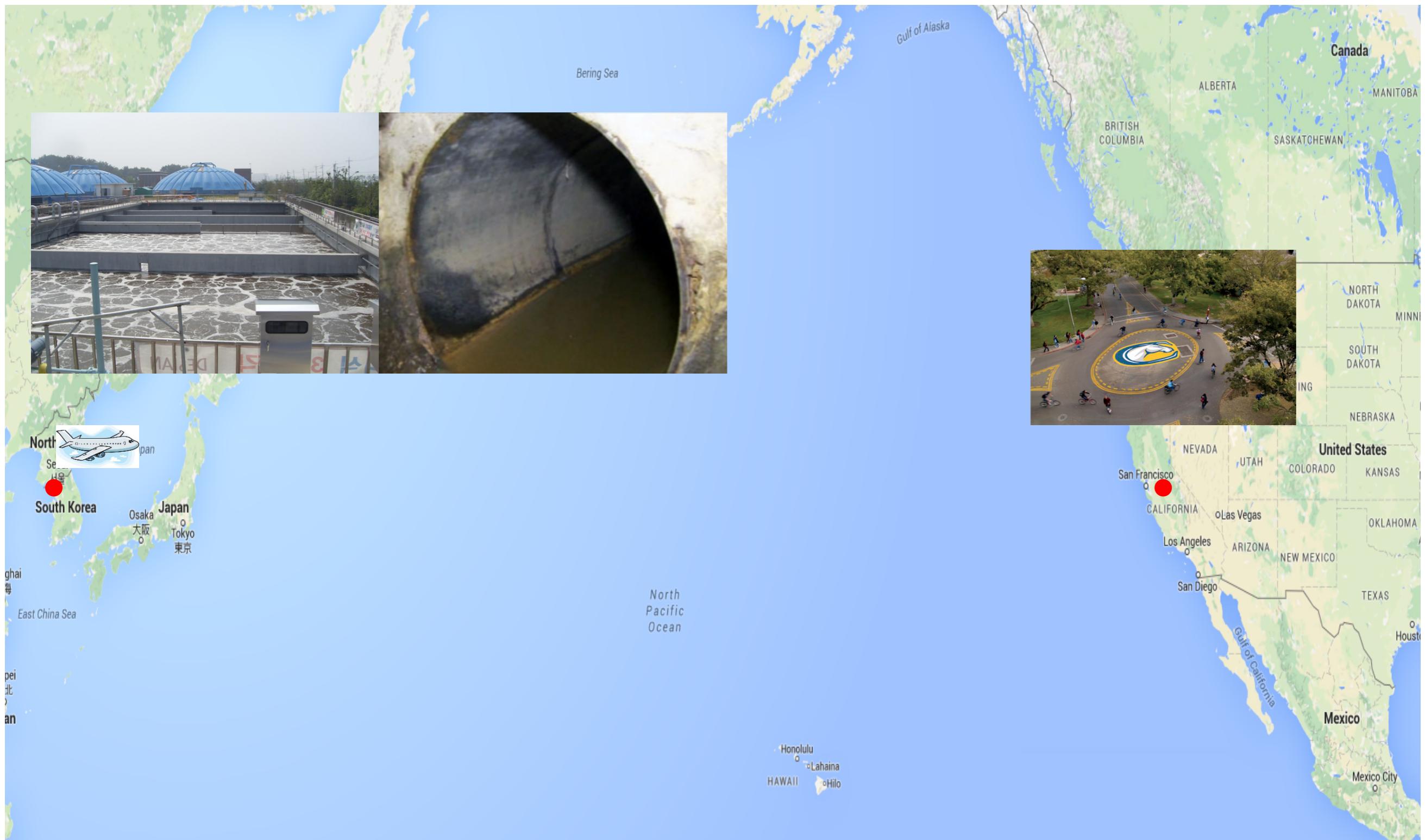


(b)

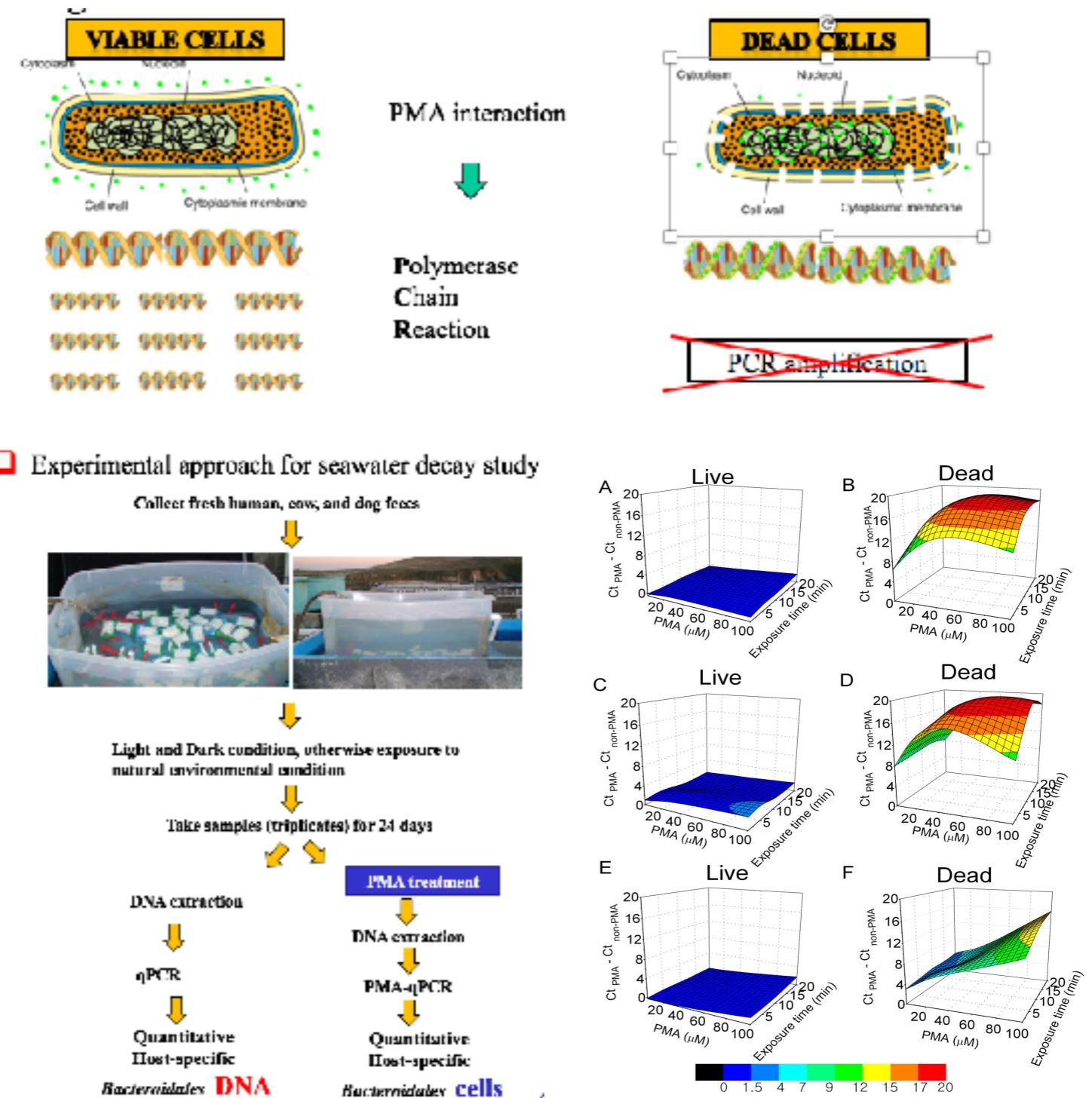
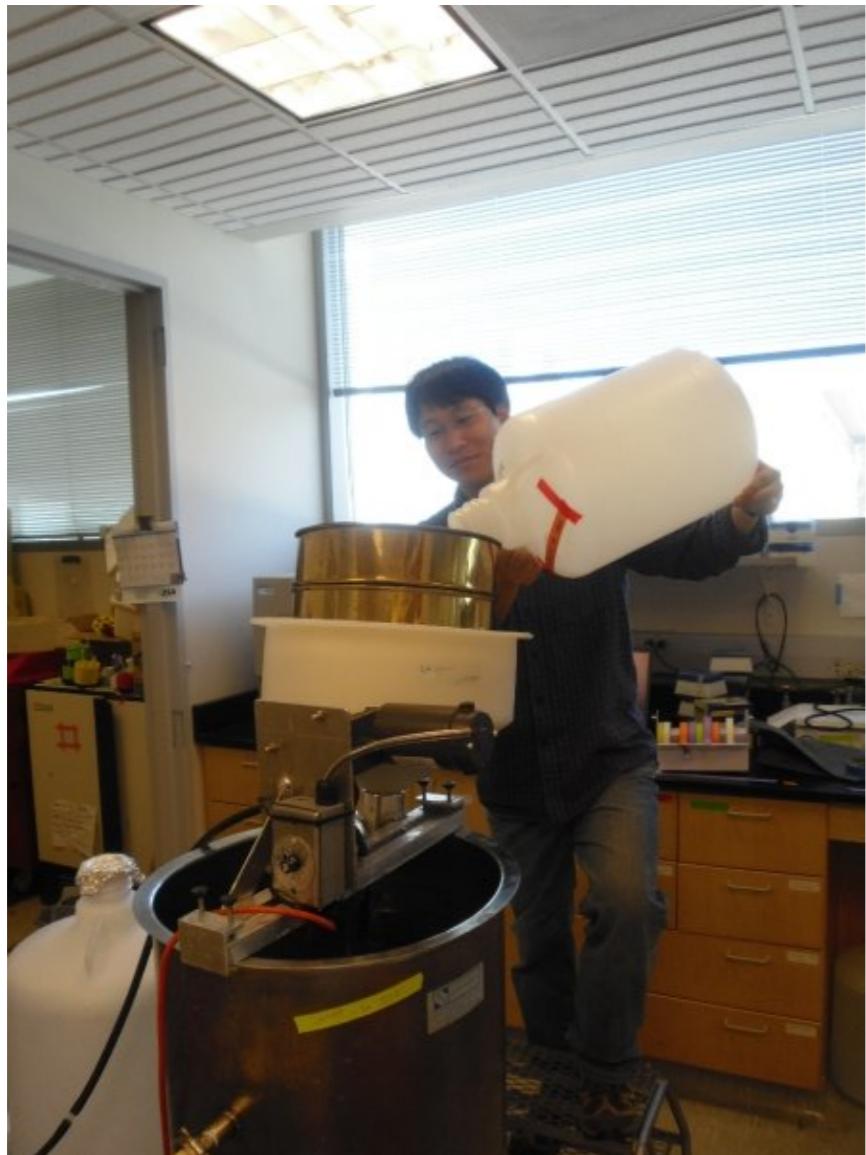


(c)

Jiri Snaidr



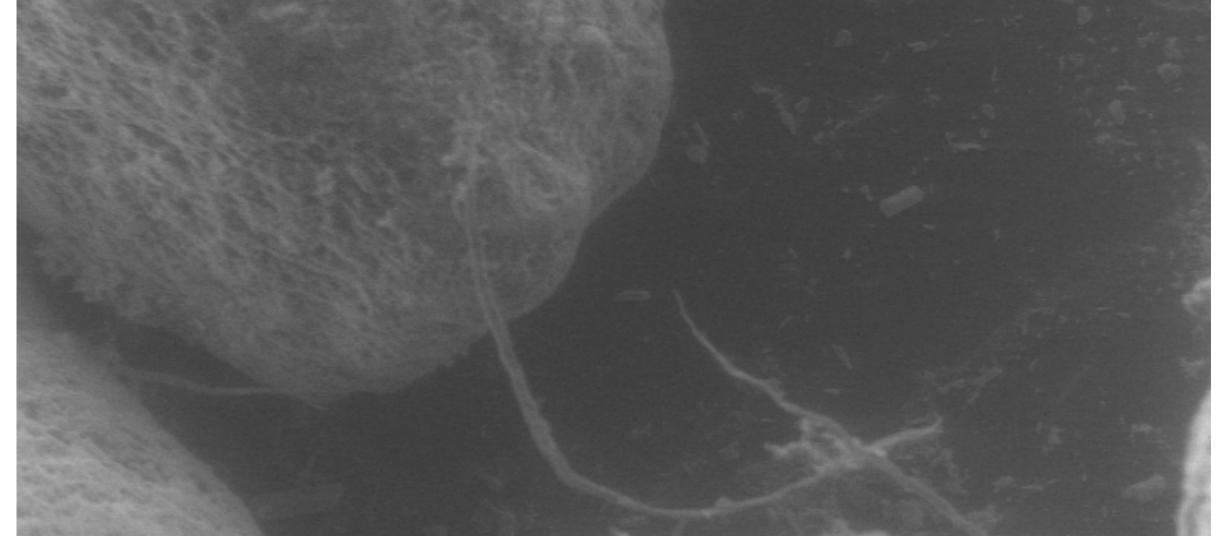
Microbiology and Molecular biology in water

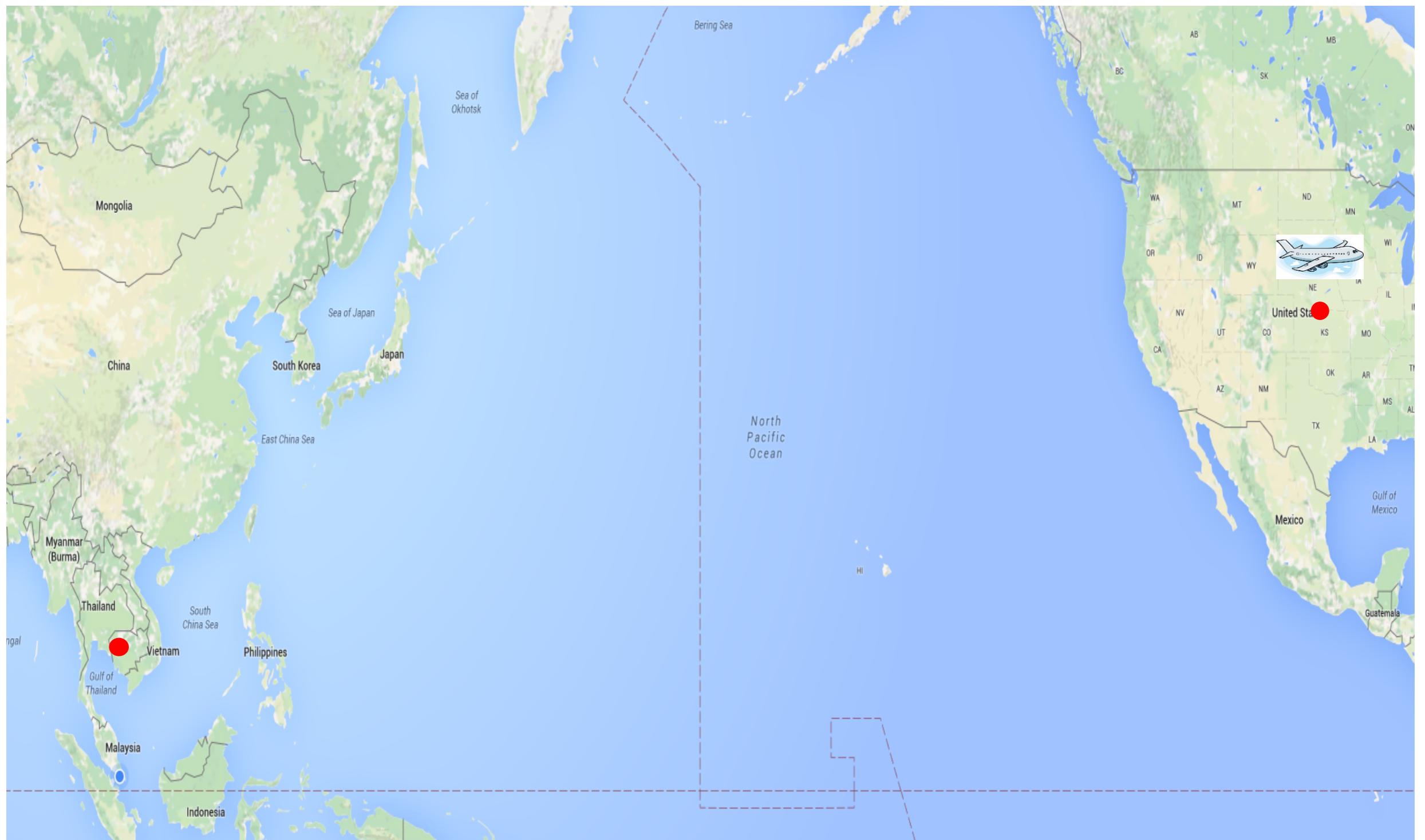




Back to Environmental Engineering

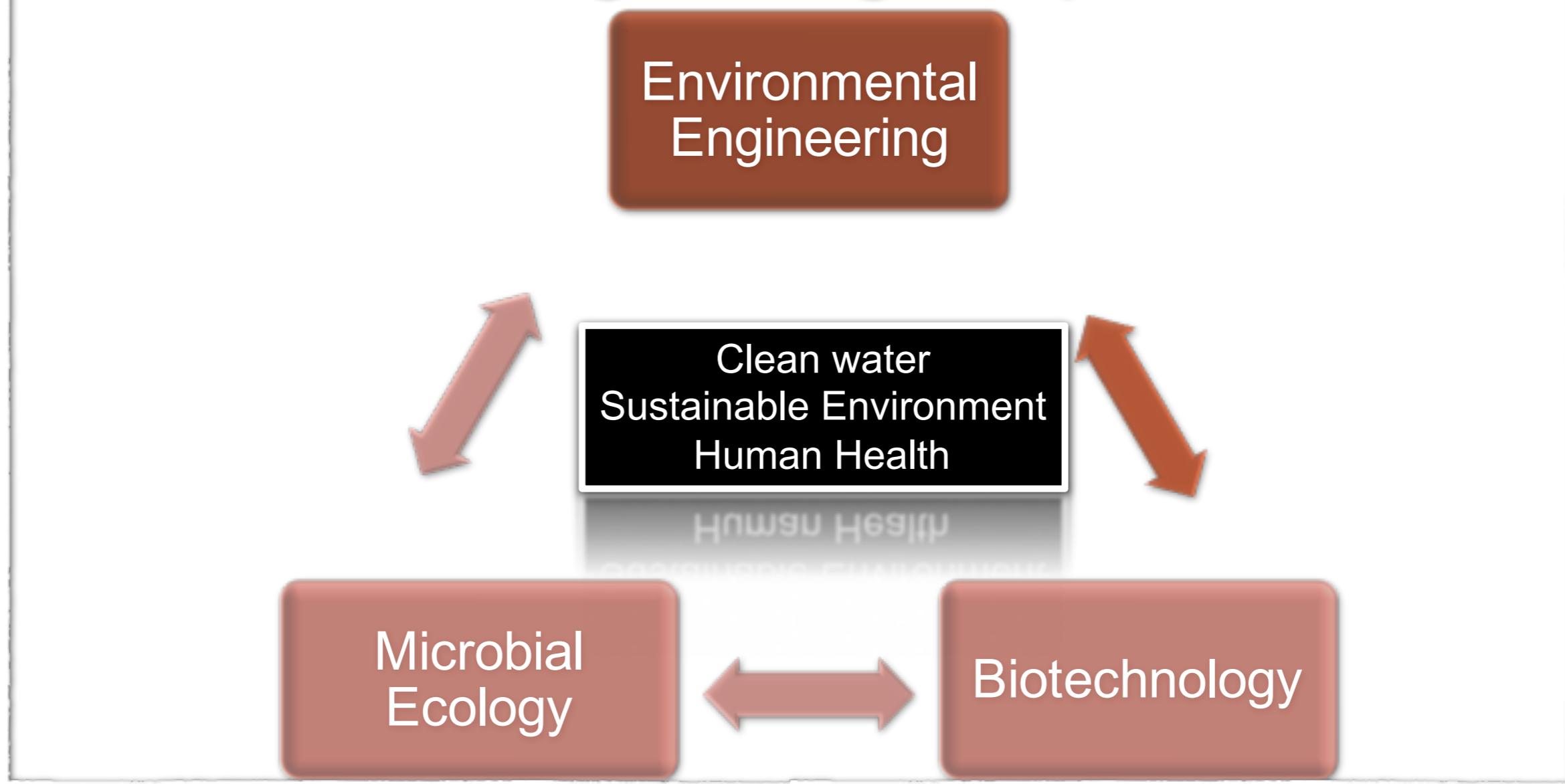
Back to Environmental Engineering





Research Theme

Understanding biological processes



Why is it important?

- The importance of microorganisms
 - Oldest form of life
 - Largest mass of living material on Earth
 - Carry out major processes for biogeochemical cycles
 - Can live in places unsuitable for other organisms
 - Other life forms require microbes to survive

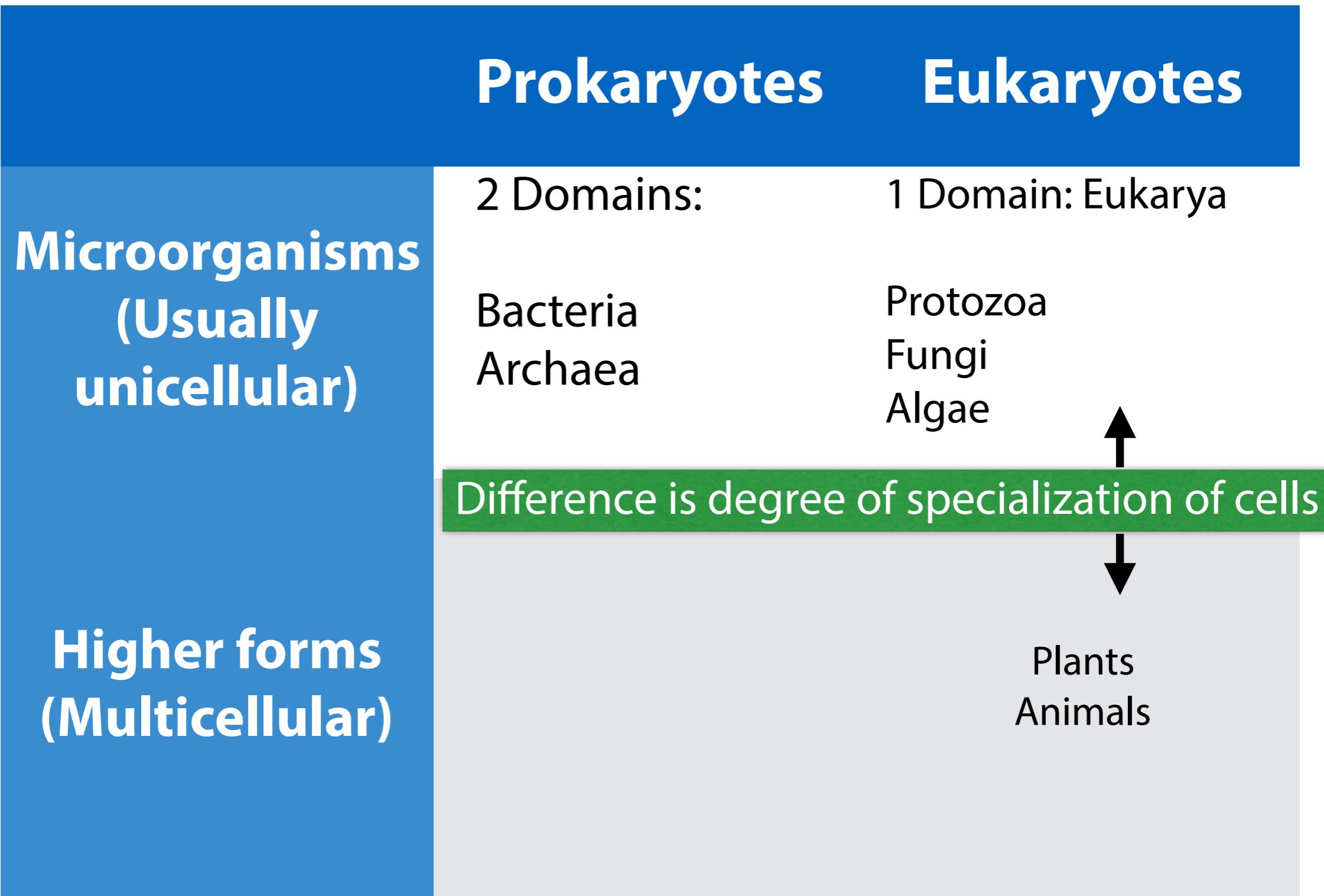
Microbiology revolves around two themes:

1. Understanding basic life processes

- Microbes are excellent models for understanding cellular processes in unicellular and multicellular organisms

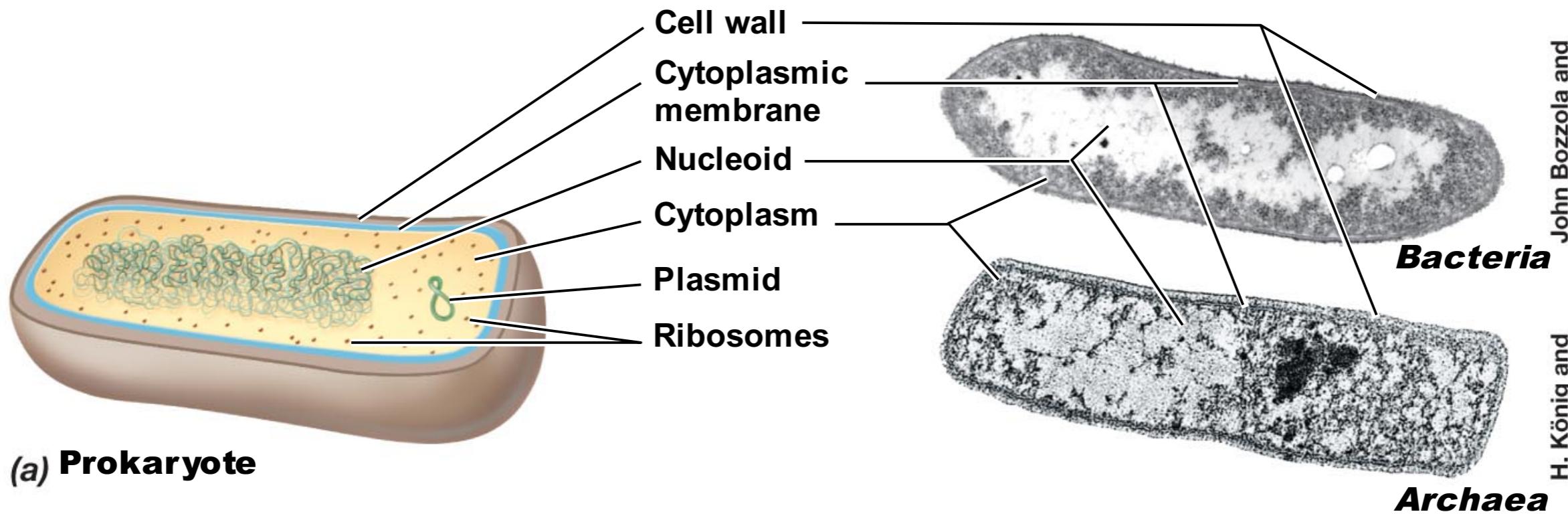
2. Applying that knowledge to the benefit of humans

- Microbes play important roles in medicine, agriculture, and industry (Environmental Eng.)



The structure of Microbial cells

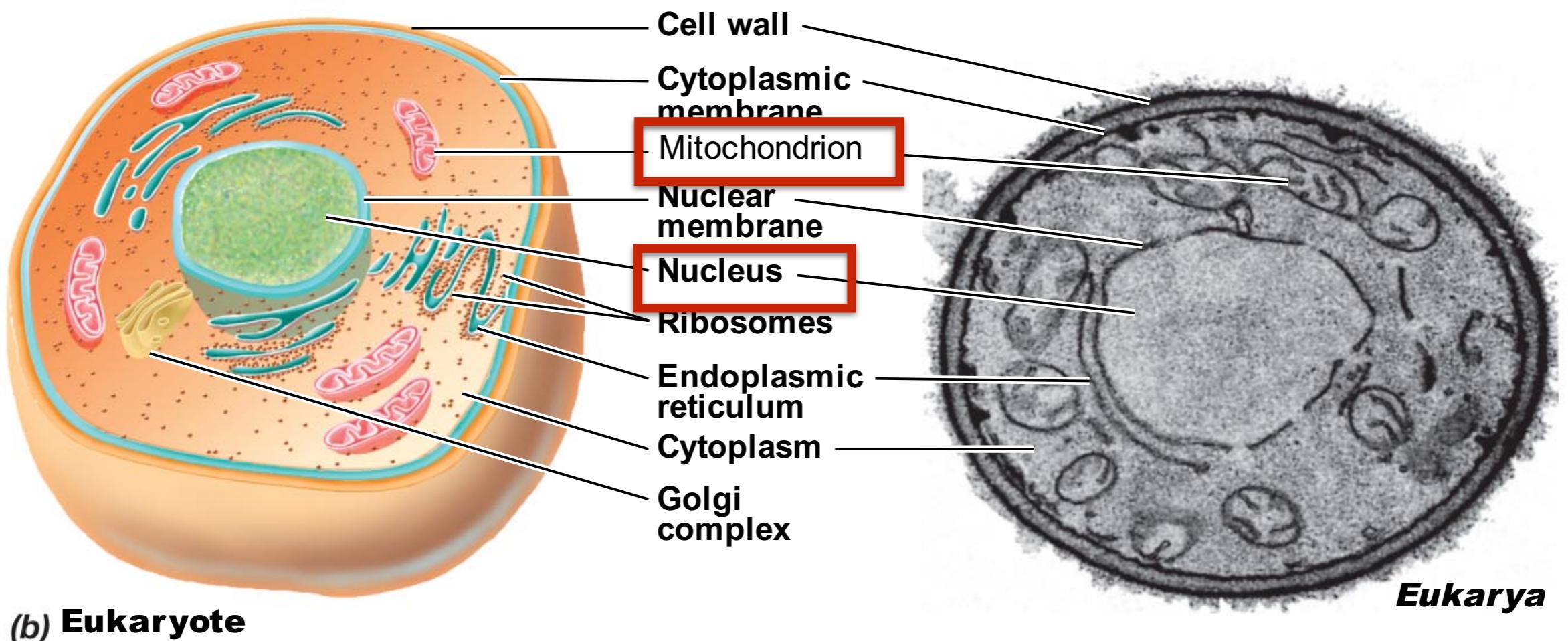
- The Cell
 - A dynamic entity that forms **the fundamental unit of life**
- Elements of microbial structure
 - All cells have the following in common:
 - **Cytoplasmic (cell) membrane** : Barrier that separates the inside of the cell from outside environment
 - **Cytoplasm** Aqueous mixture of macromolecules, ions, and ribosome
 - **Ribosomes**: Protein-synthesizing structures
 - **Cell wall** : Present in most microbes: confers structural strength
 - **Chromosome** : DNA



John Bozzola and
M.T. Madigan

H. König and
K.O. Stetter

S.F. Conti and T.D. Brock



How big is a cell?

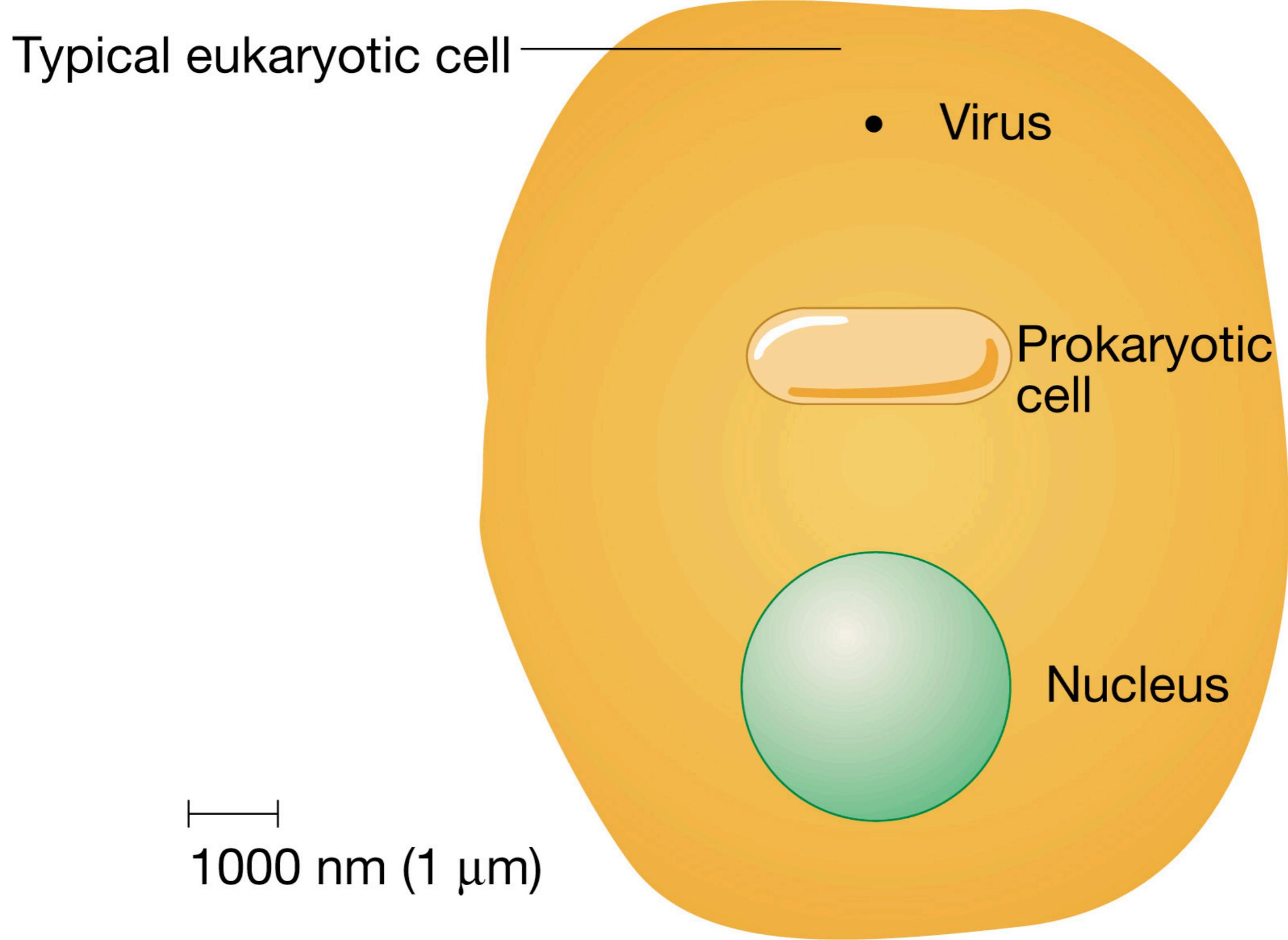
Typical prokaryotes

- $0.5 \sim 3.0 \mu\text{m}$
- 10^{-12} mL/cell
- $50 \sim 80 \% (\text{v/v})$ water
- 10^{-12} g /cell

Typical eukaryotes

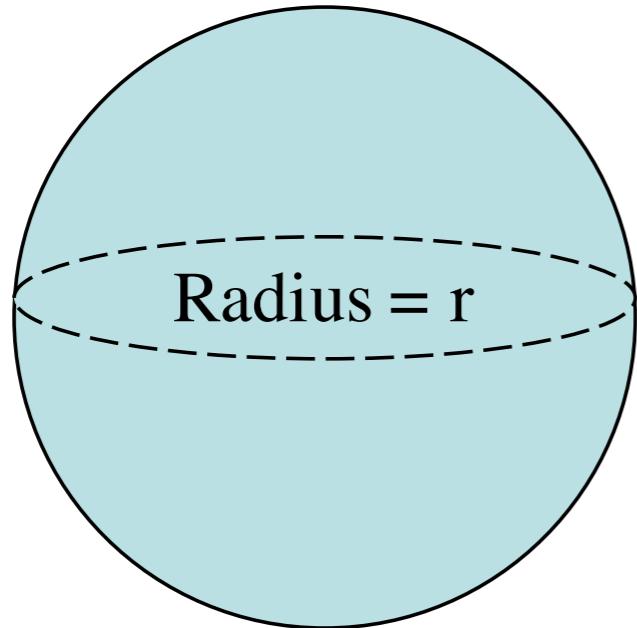
- $3 \sim 20 \mu\text{m}$
- $10^{-9} \sim 10^{-8} \text{ mL/cell}$
- $50 \sim 80 \% (\text{v/v})$ water
- $10^{-9} \sim 10^{-8} \text{ g /cell}$

Filter sterilize: $0.22 \mu\text{m}$ filter



Why is cell size important?

Surface area (SA) to volume ratio



$$\frac{SA}{Volume} = \frac{4\pi r^2}{\frac{4}{3}\pi r^3} = \frac{3}{r}$$

Smaller cells have higher SA/V ratios

- Transport rates are function of membrane surface area
 - The rates of nutrient exchanges
 - Evolution and mutation

Microorganisms outnumbers us

- On Earth

Prokaryotes: $4 - 6 \times 10^{30}$ cells*

Humans: 6×10^9 humans*

- In/on the human body

Prokaryotic cells: 7×10^{13} *

Human cells: $1-10 \times 10^{13}$



*Whitman, W.B., D.C. Coleman, and W.J.Wiebe. 1993. "Prokaryotes: The Unseen Majority" *PNAS* 95: 6578-6583.

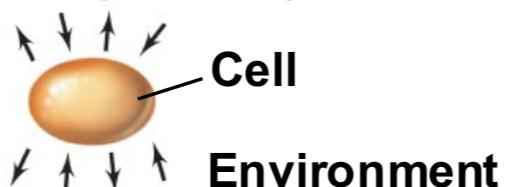
Characteristics of living cells

Properties of *all* cells:

Metabolism

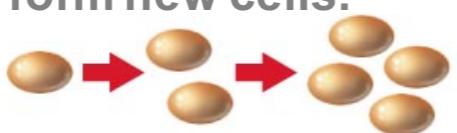
Cells take up nutrients, transform them, and expel wastes.

1. **Genetic** (replication, transcription, translation)
2. **Catalytic** (energy, biosyntheses)



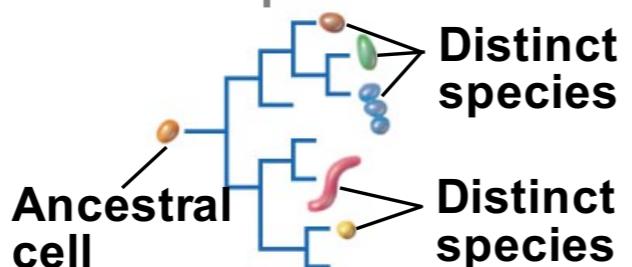
Growth

Nutrients from the environment are converted into new cell materials to form new cells.



Evolution

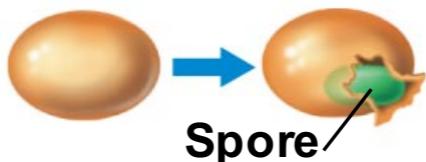
Cells evolve to display new properties. Phylogenetic trees capture evolutionary relationships.



Properties of *some* cells:

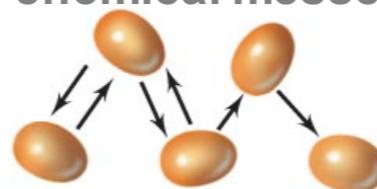
Differentiation

Some cells can form new cell structures such as a spore.



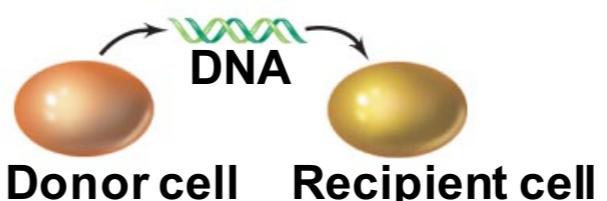
Communication

Cells interact with each other by chemical messengers.



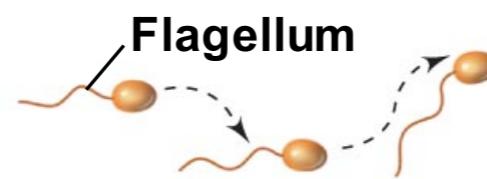
Genetic exchange

Cells can exchange genes by several mechanisms.

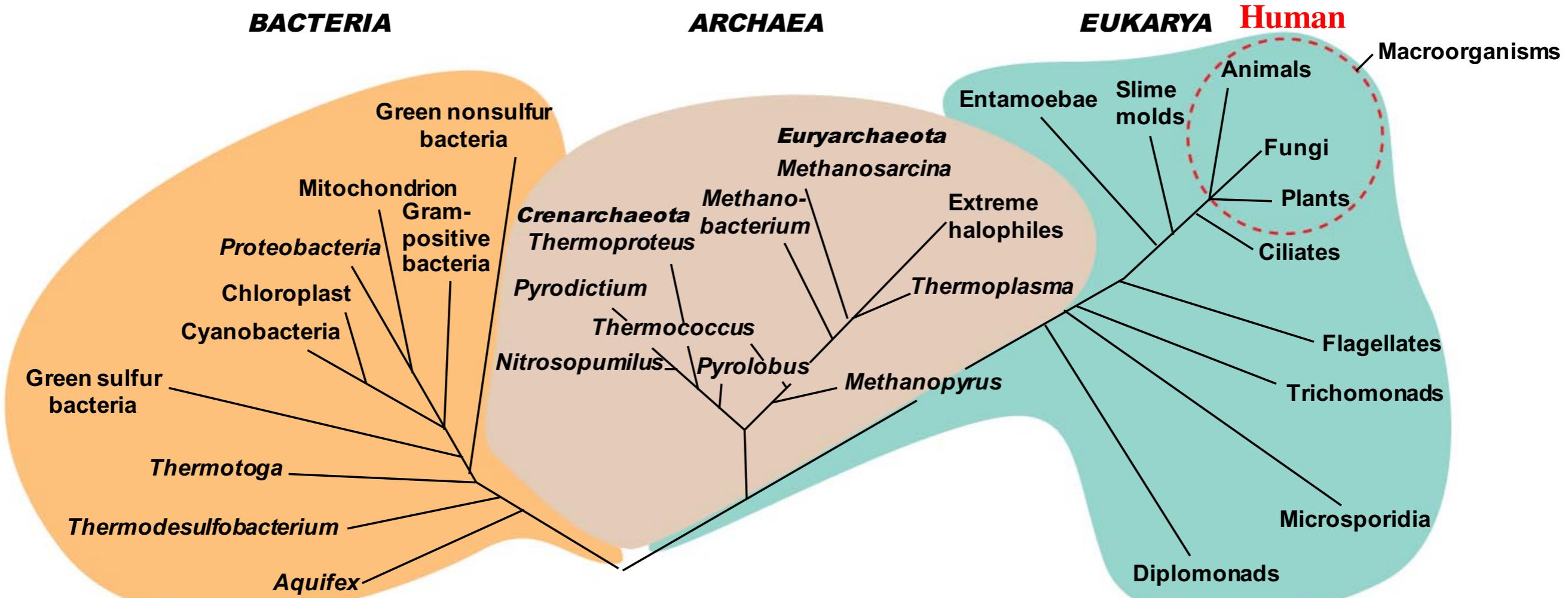


Motility

Some cells are capable of self-propulsion.



Evolution and Diversity of microbial cells



(b)

Ribosomal RNA gene phylogenies

Microorganisms and their environment

- Diversity and abundances of microbes are controlled by **resources (nutrients)** and **environmental conditions** (e.g., temp, pH, O₂)
- The activities of microbial communities can affect the **chemical and physical properties of their habitats**
- Microbes also interact with their physical and chemical environment
- Ecosystems are greatly influenced (if not controlled) by **microbial activities**
 - Microorganisms change the chemical and physical properties of their habitats through their activities
 - For example, removal of nutrients from the environment and the excretion of waste products

Where do microorganisms exist?

Anywhere their requirements are met:

Water

Carbon source

Nitrogen, phosphorus, trace nutrients

Energy source

They are almost everywhere

Soil

Hot springs

Fresh water, salt water

Glaciers/Antarctica

Except

Lava*

Extreme pH

$\text{pH} < 0$, $\text{pH} > 12$



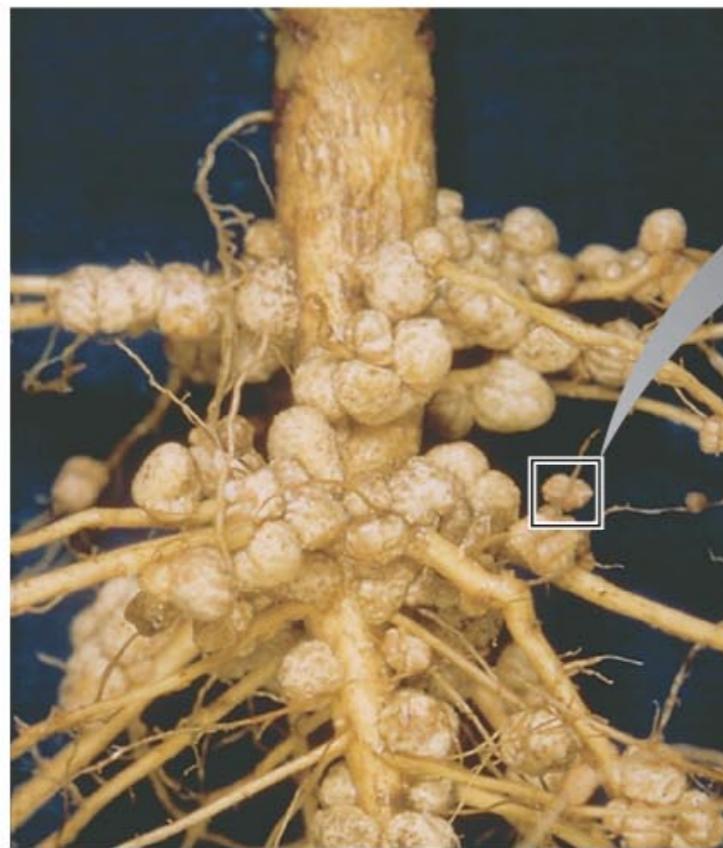
Yellowstone National Park, Kirisits, 2000.

acidophiles and thermophiles

The impact of microorganisms on Humans

Microorganisms and agriculture

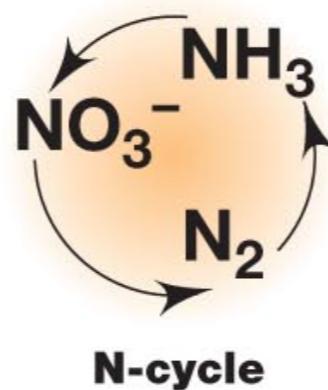
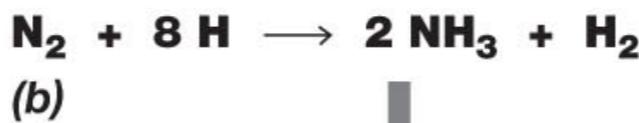
- Many aspects of agriculture depend on microbial activities
 - Positive impacts
 - Nitrogen-fixing bacteria
 - Cellulose-degrading microbes in the rumen
 - Regeneration of nutrients in soil and water
 - Negative impacts
 - Diseases in plants and animals



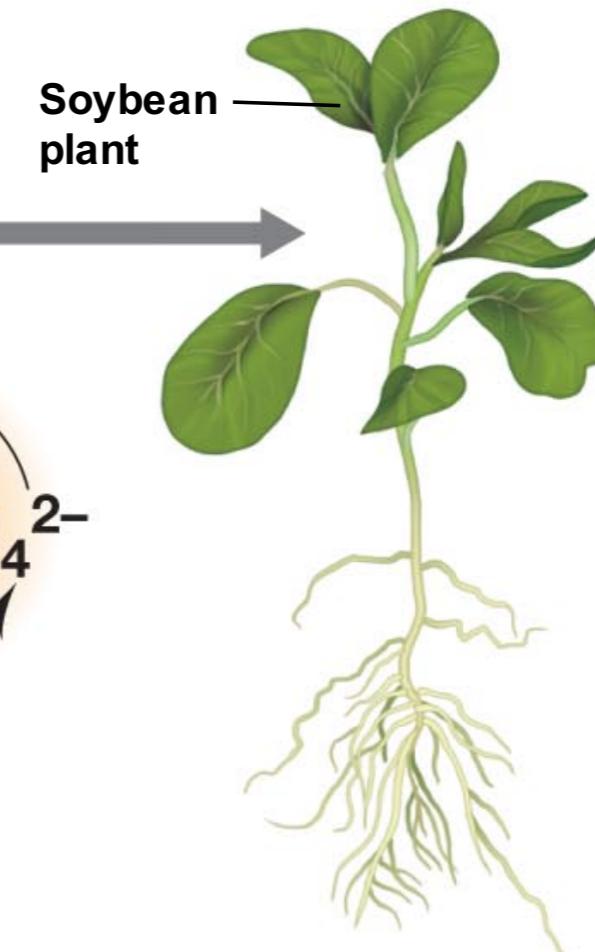
(a)



(d)



(c)



Rumen

Grass → Cellulose → Glucose → Microbial fermentation

Fatty acids
(Nutrition for animal)

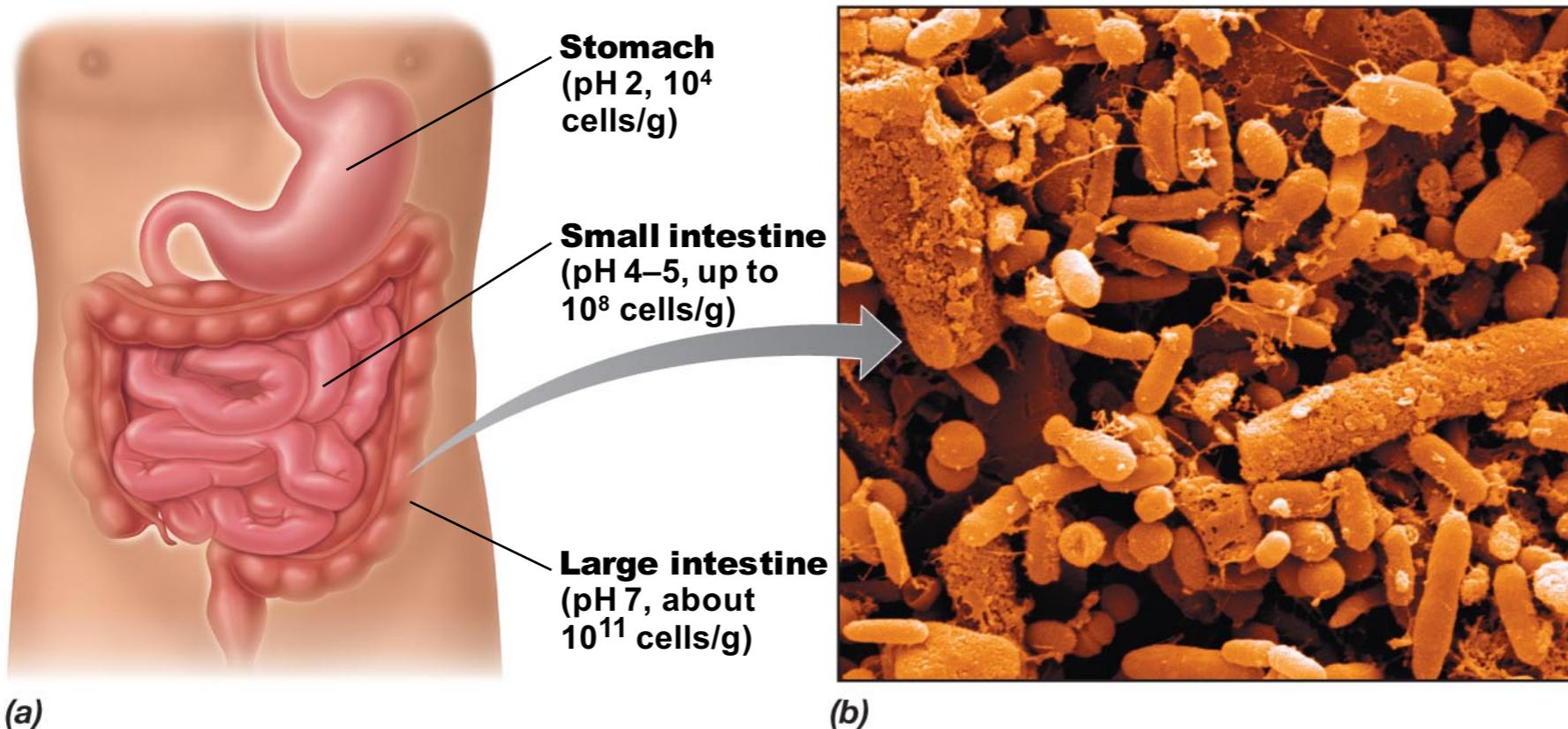
$CO_2 + CH_4$
(Waste products)

2015, Pearson Education Inc

The impact of microorganisms on Humans

Microorganisms and human gastrointestinal (GI) tract

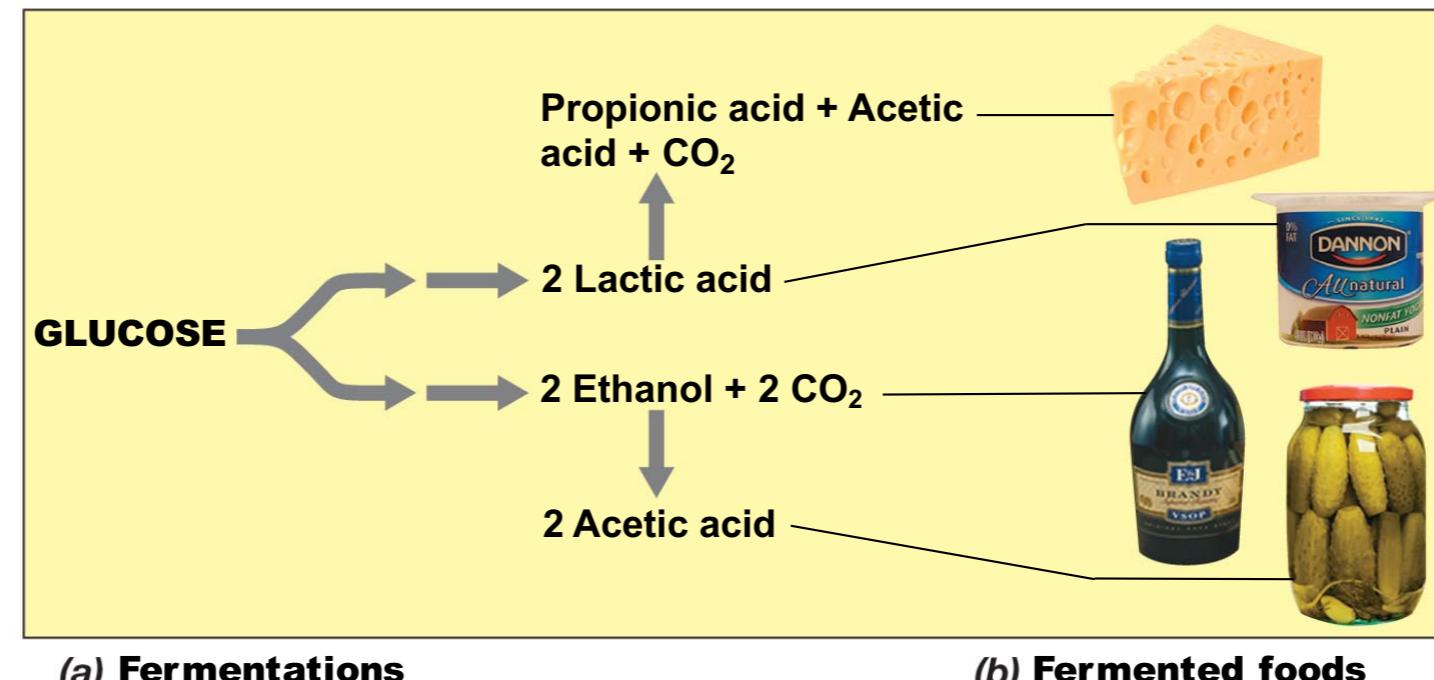
- High numbers of microorganisms occur in colon and oral cavity
 - Positive impacts
 - Synthesize vitamins and other nutrients
 - Compete with pathogens for space and resources



The impact of microorganisms on Humans

Microorganisms and Food

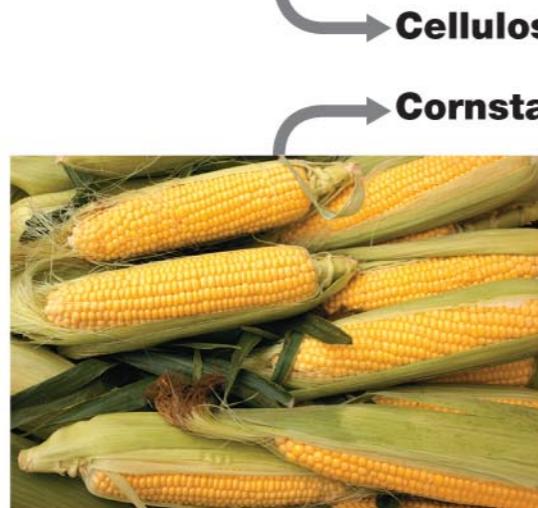
- Negative impacts
 - Microorganisms can cause food spoilage; for many foods, methods of preservation are needed
- Positive impacts
 - Microbial transformations (typically fermentations) yield
 - Dairy products (e.g., cheeses, yogurt, buttermilk)
 - Other food products (e.g., sauerkraut, pickles, leavened breads, beer)



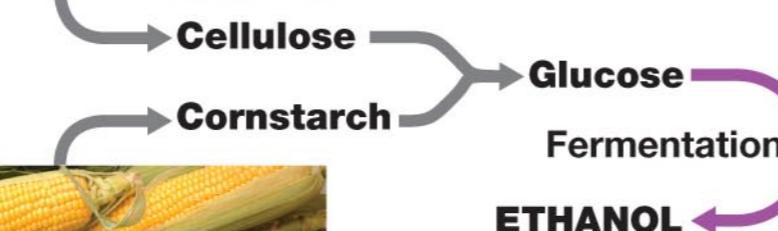
The impact of microorganisms on Humans

Microorganisms, Energy, and environments

- The role of microbes in production of biofuels
 - For example, methane, ethanol, hydrogen
- The role of microbes in cleaning up pollutants (bioremediation)



(a)

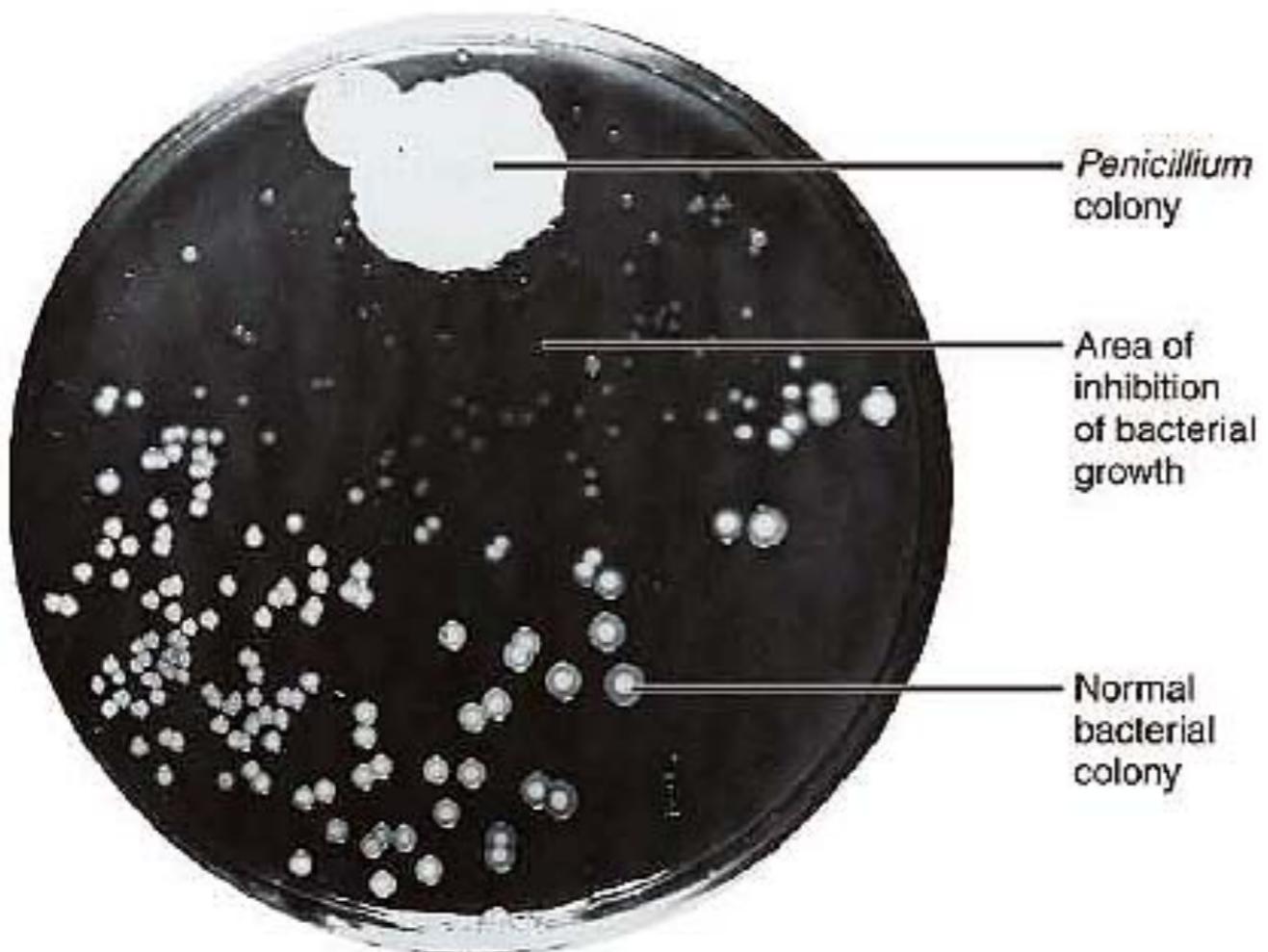


(b)

The impact of microorganisms on Humans

Microorganisms and their genetic resources

- Exploitation of microbes for production of antibiotics, enzymes, and various chemicals
- Genetic engineering of microbes to generate products of value to humans, such as insulin (biotechnology)

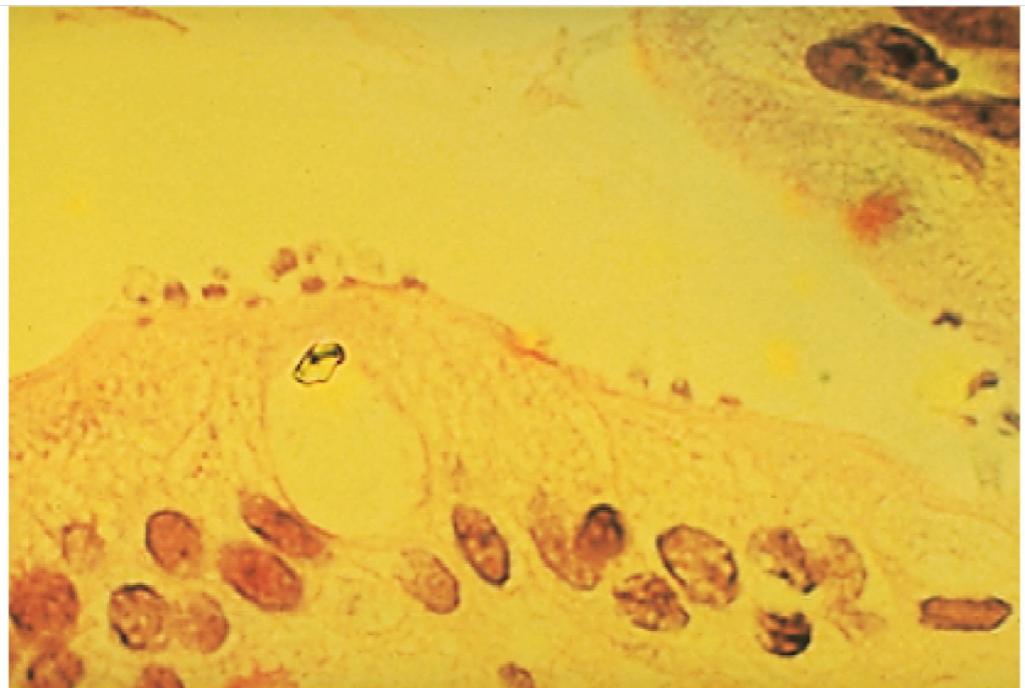


**Source: Alexander Fleming,
"On the Antibacterial Action of
Cultures of a Penicillium, with
Special Reference to Their Use
in the Isolation of *B. Influenzae*"
*The British Journal of
Experimental Pathology*, (1929)
x: p.226.**

Why do environmental engineers care about microbiology?

I. Harmful microorganisms

A. Pathogens



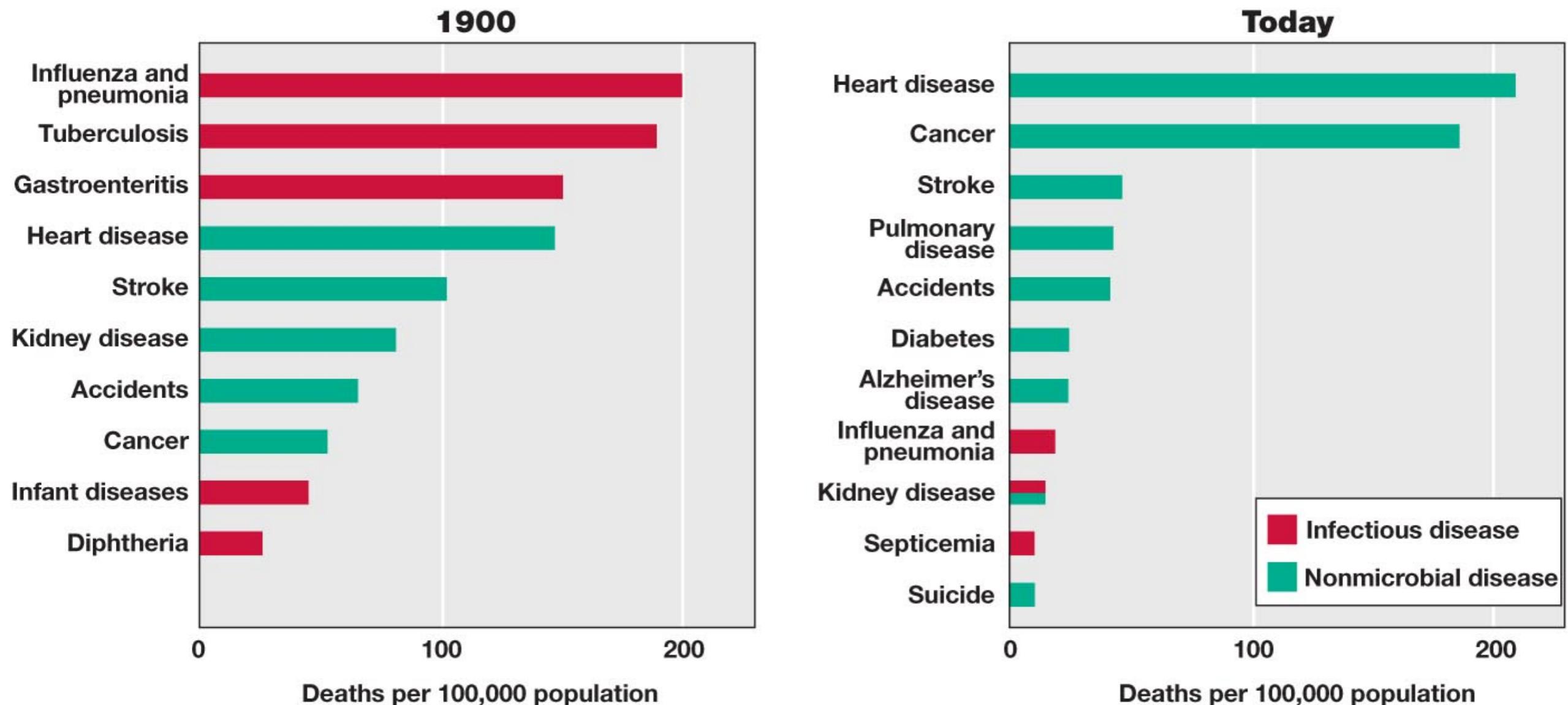
Centers for Disease Control



www.geocities.com/CapeCanaveral/3504/gallery.htm

Cryptosporidium (cryptosporidiosis)

E. coli (gastroenteritis)



Change between 1900 and 2000:

Understanding of disease processes
Better sanitation and public health practices
Use of antimicrobials
Active of vaccination

Most common Waterborne Diseases of Concern

Viral hepatitis (hepatitis A)

Viral gastroenteritis (Norwalk agent, rotavirus)

Campylobacteriosis (*Campylobacter jejuni*)

Cholera (*Vibrio cholerae*)

Shigellosis (*Shigella* spp.)

Salmonellosis (*Salmonella* spp.)

Typhoid fever (*Salmonella typhi*)

Amebiasis (*Entamoeba histolytica*)

Giardiasis (*Giardia lamblia*)

Cryptosporidiosis (*Cryptosporidium parvum*)

Too many waterborne pathogens to count!

How do we typically monitor pathogens in water

- Use an indicator organism
 - Fecal coliform (FC)
 - Live in the intestines of warm-blooded animals
 - Typically not pathogenic
 - Example: E. coli
 - What does their presence indicate?
Fecal contamination
Potential presence of pathogens

Eutrophication

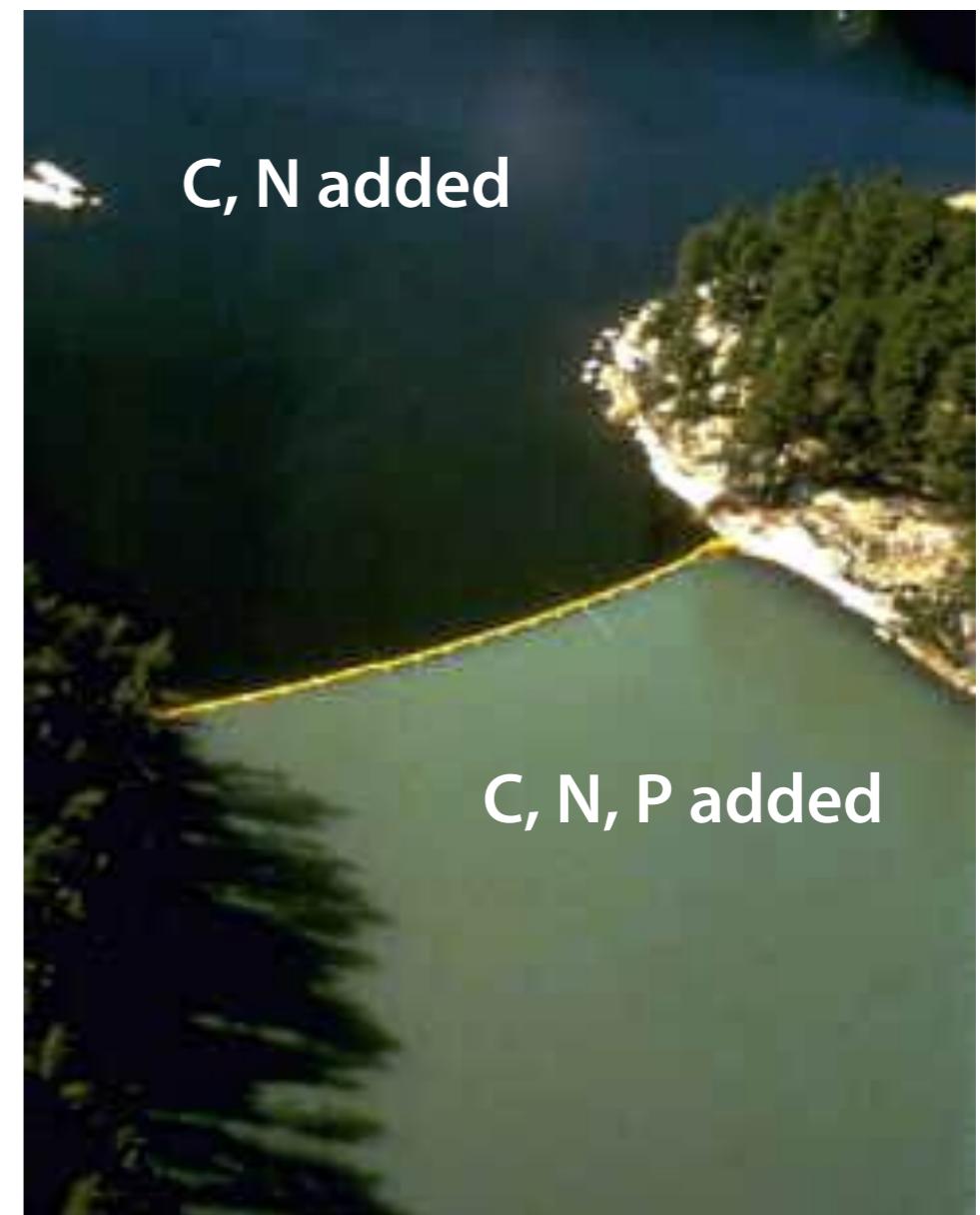
- Water bodies receive excess nutrients (N, P)
 - Growth of cyanobacteria, algae, aquatic weeds

Their decomposition consumes O₂
Cyanobacteria produce neurotoxins
and taste & odor compounds

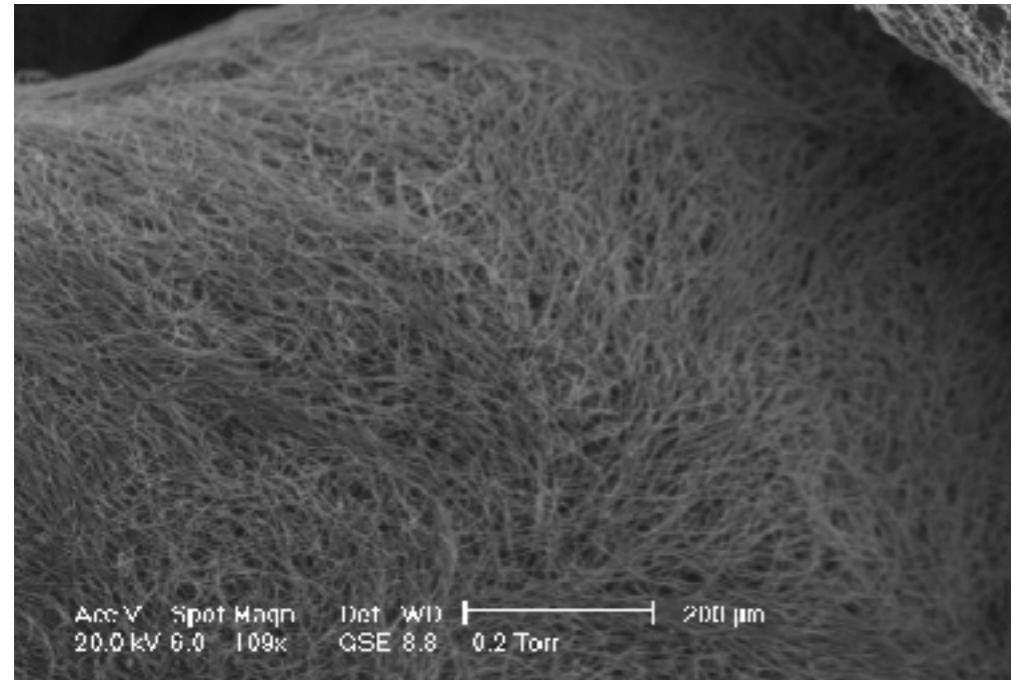


Kranji Reservoir

<http://wildshores.blogspot.sg/>



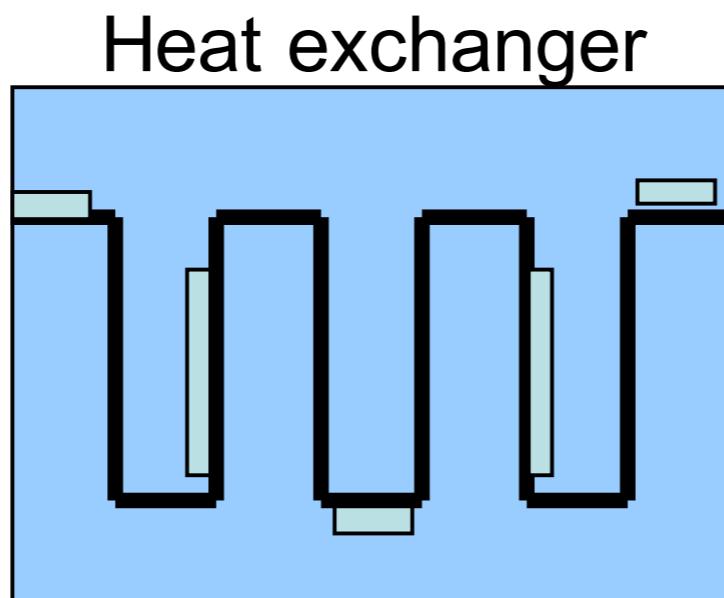
Surface fouling by biofilms



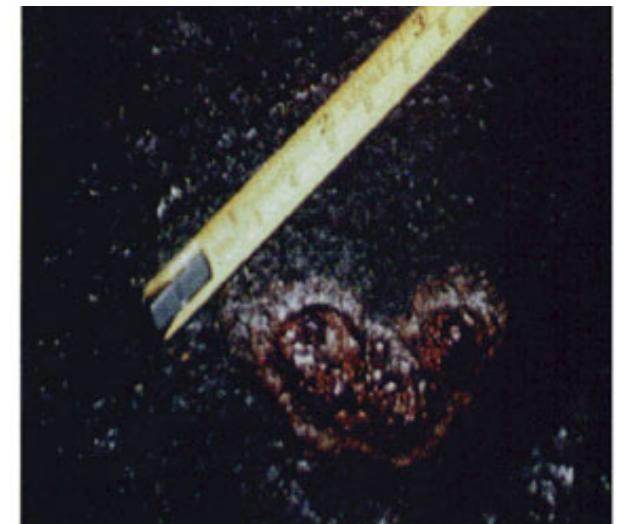
Biofilm: Assemblage of microorganisms
Encased in extracellular matrix
Attached to solid surface

GAC Biofilter, Bae, 2013

Biofilms have low thermal conductivity.



Ship hull corrosion



Why do environmental engineers care about microbiology?

II. Beneficial microorganism

A. Biodegradation of organic / inorganic wastes

1. In situ bioremediation of soils / ground water

a. Accelerated in situ bioremediation: add substrate , nutrients, or bacteria

b. Natural attenuation: without human interventions

2. Wastewater treatment plants: biological treatment is the cornerstone

a. Activated sludge

b. Trickling filters

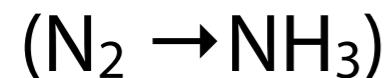
3. Drinking water treatment plants: Biological treatment is becoming more popular

Why do environmental engineers care about microbiology?

II. Beneficial microorganism (continues)

B. microbial nutrient cycling

1. N fixation



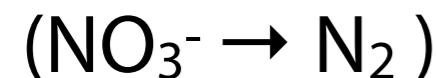
3. Assimilation



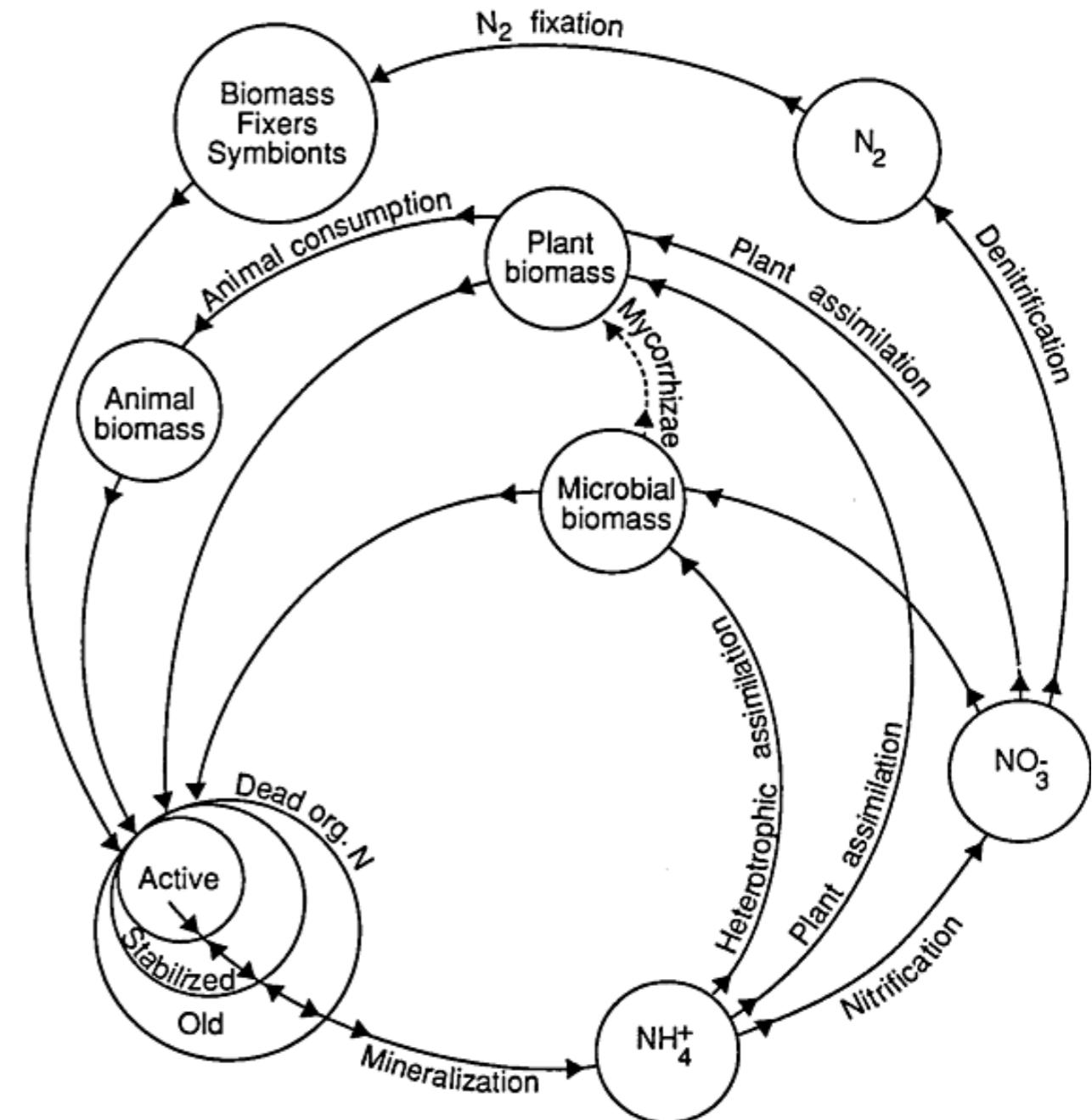
5. Nitrification



7. Denitrification



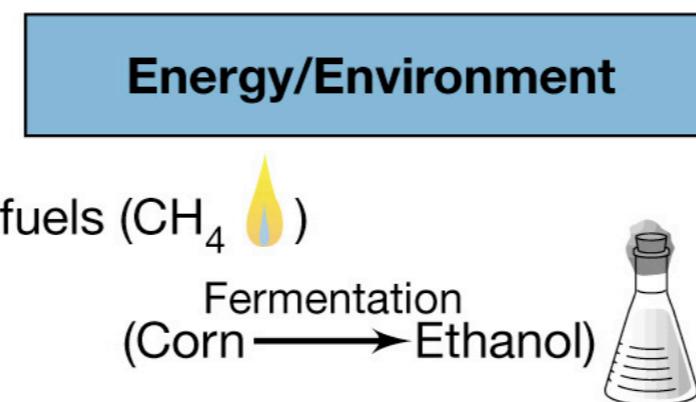
9. Ammonification



Why do environmental engineers care about microbiology?

II. Beneficial microorganism (continues)

B. Other Beneficial microorganism



Lipid-accumulating algae

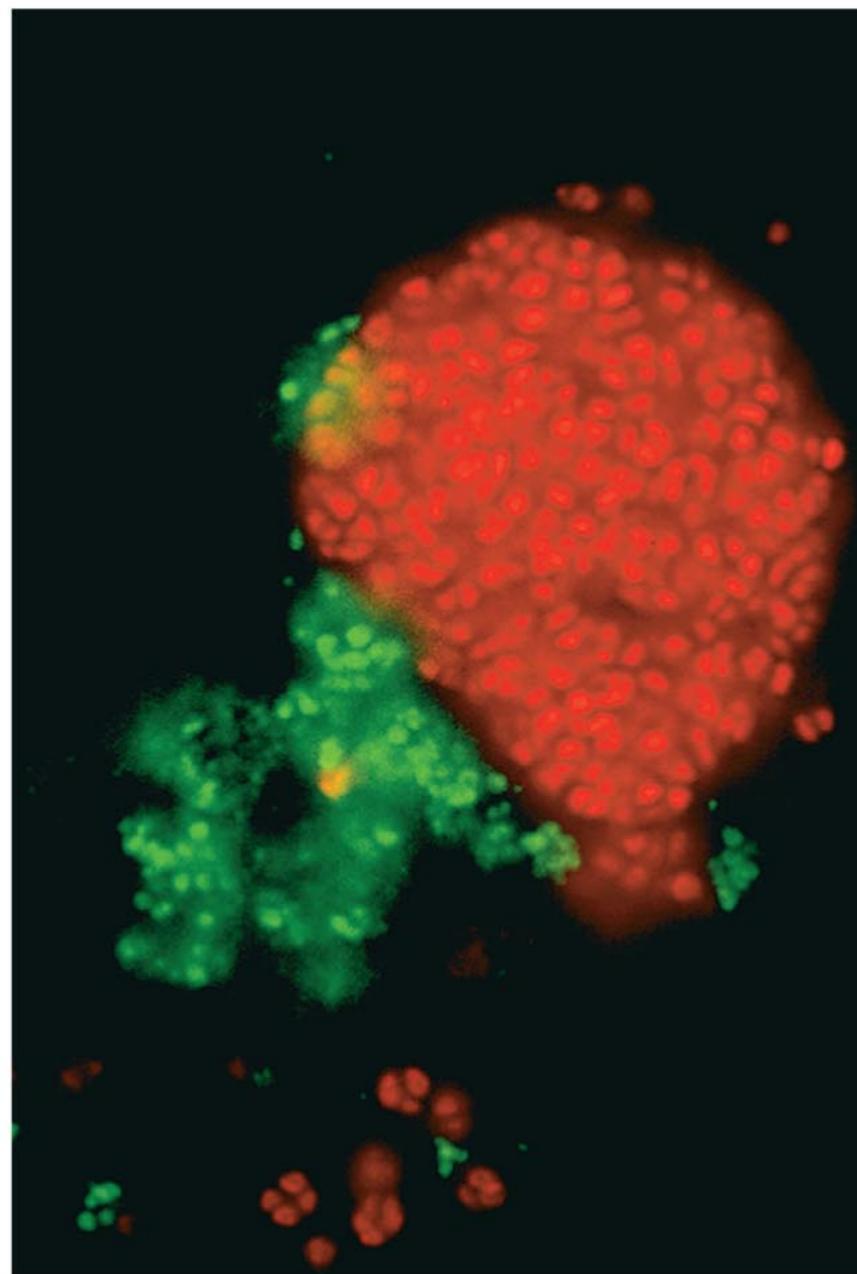


Modern Environmental Microbiology



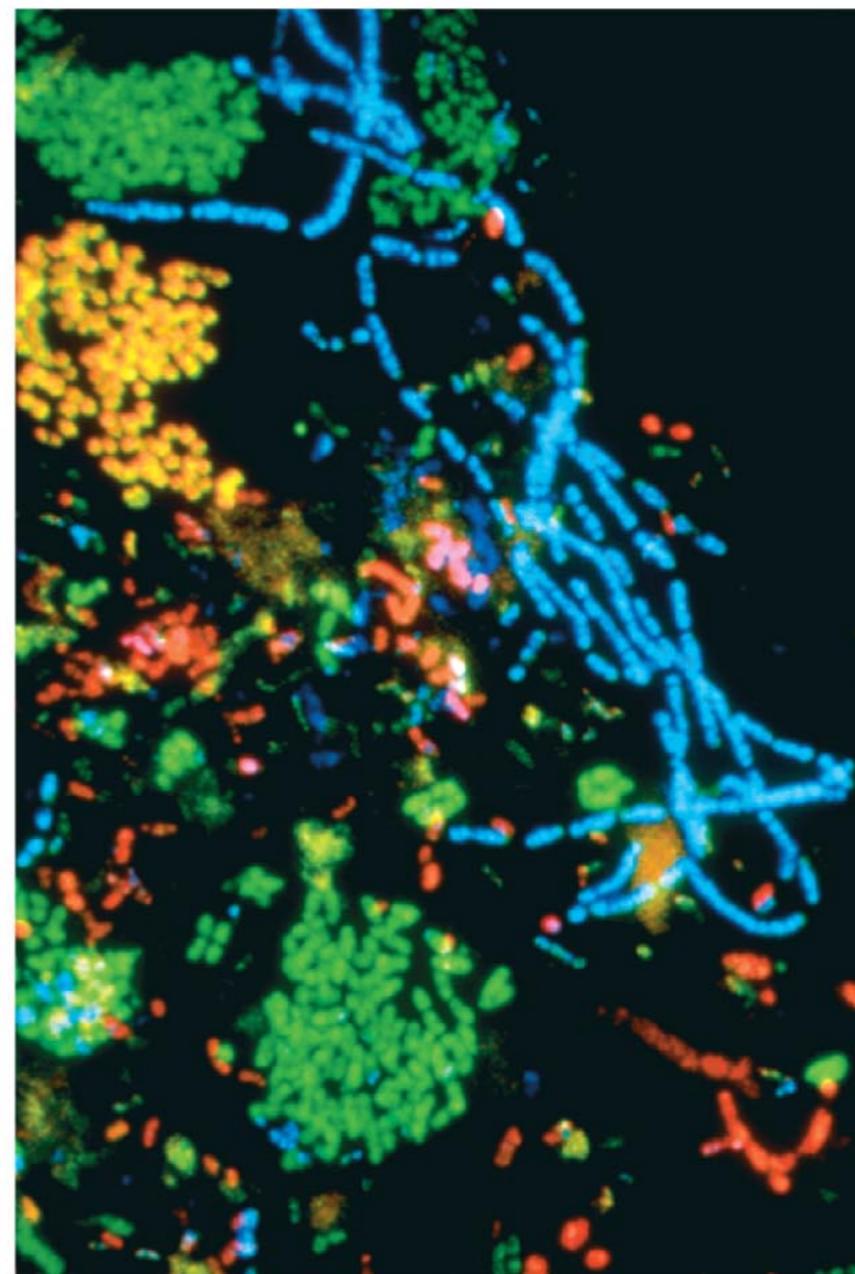
FIGURE 1.1 Environmental microbiology interfaces with many other fields of microbiology.

Molecular Biological Tools for Environmental Engineerings



(a)

Michael Wagner and David A. Stahl

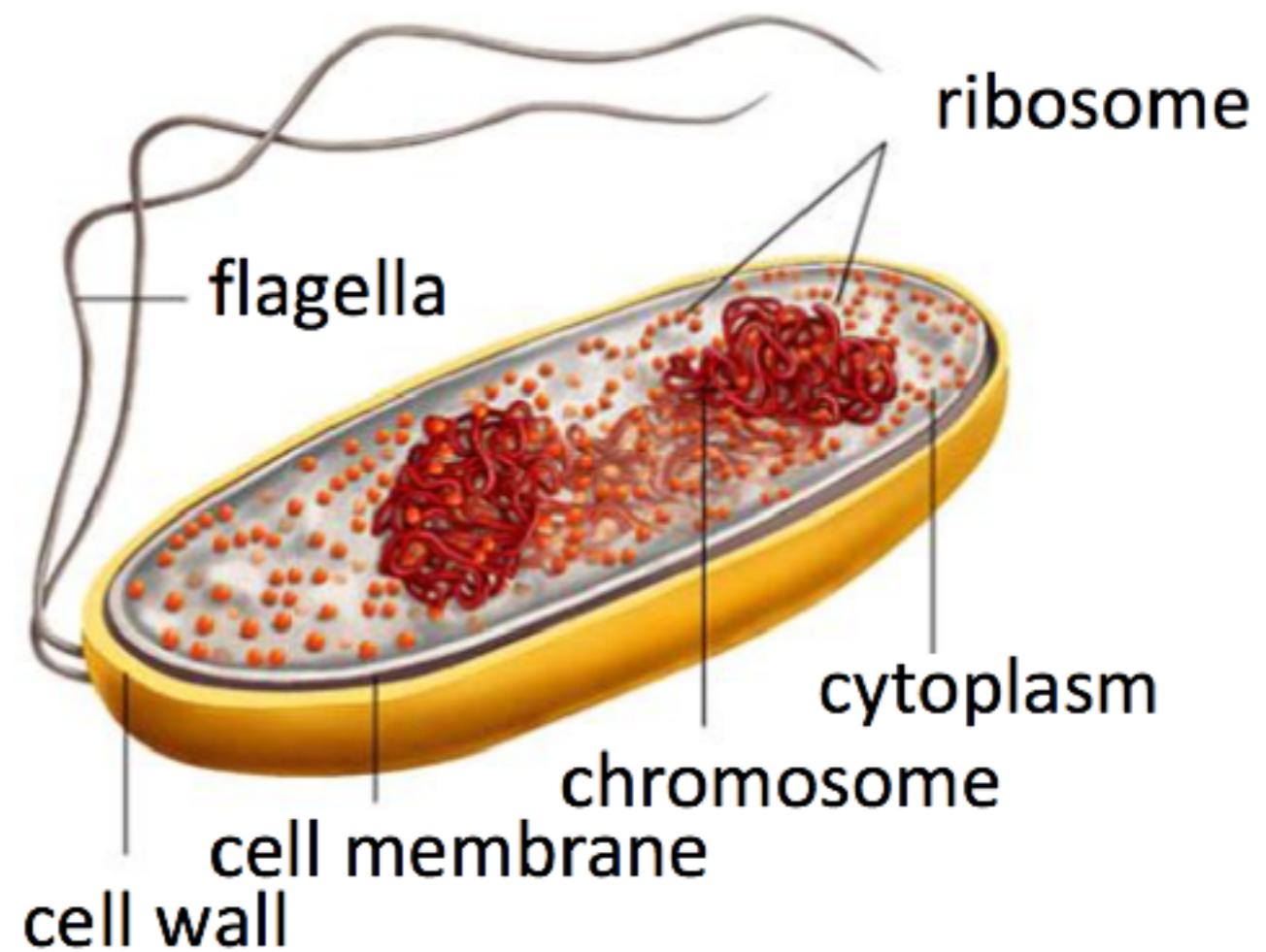


(b)

Michael Wagner and Jiri Snajdr

Questions to be Answered in a Microbial Ecology Study

- Five Ws and One H
 - Who was involved?
 - What happened?
 - Where did it take place?
 - When did it take place?
 - Why did it happen?
 - How did it happen?



Exercise

- Fill out the following table for biological nitrification in WWTP

Five Ws and one H

Microbial Process

Who was involved

What happened

Where did it take place

When did it take place

Why did it happen

How did it happen

Methods for understanding biological processes

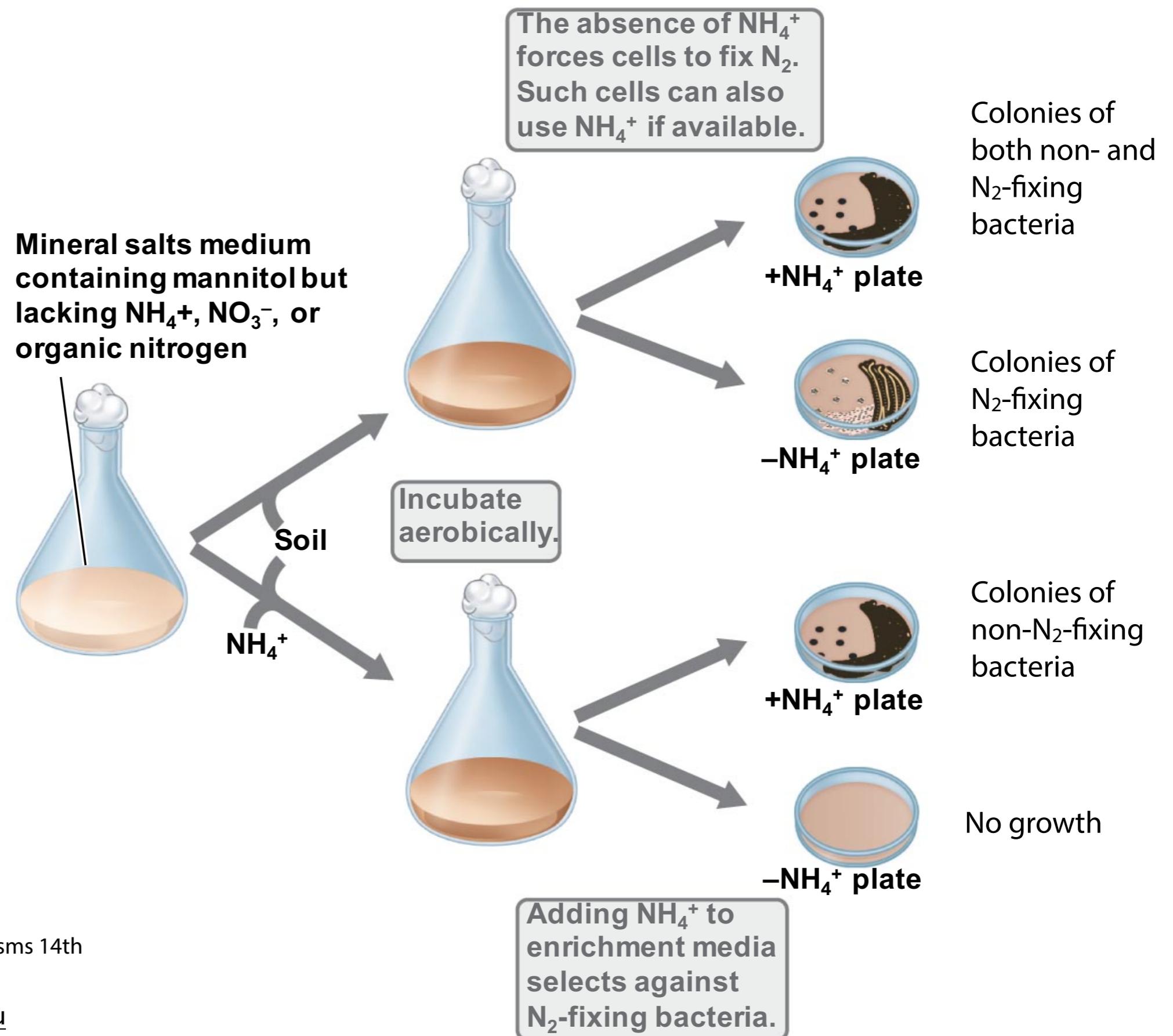
- Fill out the following table for biological nitrification in WWTP

Five Ws and one H	Microbial Process
Who was involved	Nitrifies, ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB)
What happened	NH_4^+ oxidized to NO_3^-
Where did it take place	In the aeration tank where O_2 is $> 1 \text{ mg/L}$
When did it take place	When the SRT > 15 days
Why did it happen	NH_4^+ and NO_2^- are electron donors and transfer electrons to electron acceptors O_2
How did it happen	$\text{NH}_4^+ \rightarrow \text{NO}_2^- \text{ (AOB)}$ & $\text{NO}_2^- \rightarrow \text{NO}_3^- \text{ (NOB)}$

Culture-Dependent Analyses

- Isolation
 - The separation of individual organisms from the mixed community
- Enrichment cultures
 - Selection from desired organisms through manipulation of medium and incubation conditions
- Inoculum
 - The sample from which microorganisms will be isolated

The isolation of *Azotobacter*, N₂ fixation bacteria



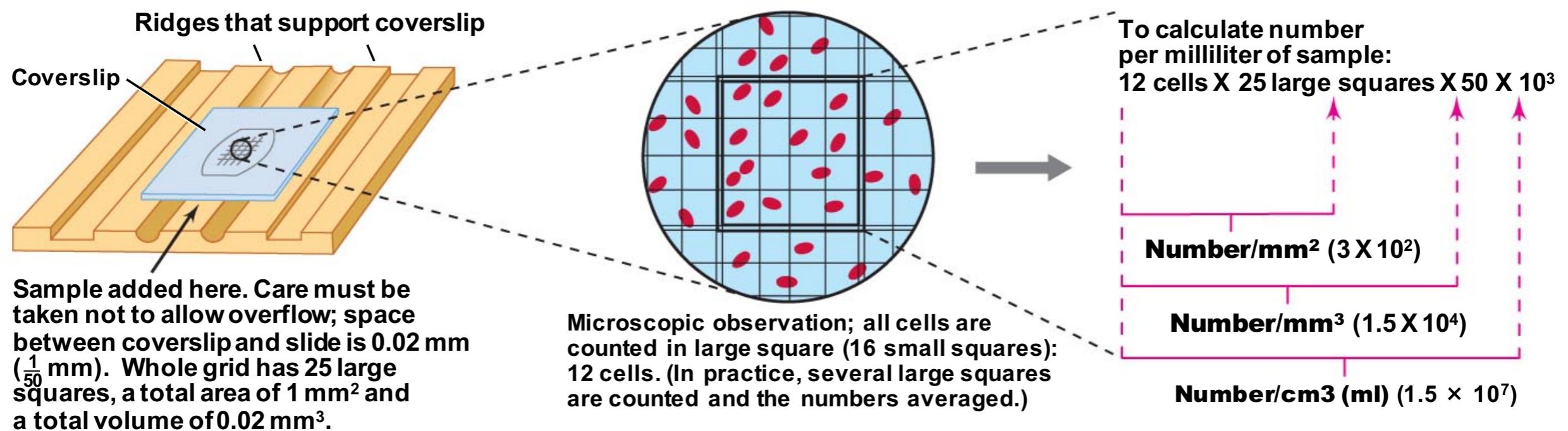
Enrichment

- **Enrichment cultures**
 - Can prove the presence of an organisms in a habitat
 - Cannot prove that an organism does not inhabit an environment
- The ability to isolate an organisms from an environment says nothing about its **ecological significance**
- **Enrichment bias**
 - Microorganisms cultured in the lab are frequently only minor components of the microbial ecosystem
 - Reason: the nutrients available in the lab culture are typically much higher than in nature
 - Dilution of inoculum is performed to eliminate rapidly growing, but quantitatively insignificant, weed species

Enumeration

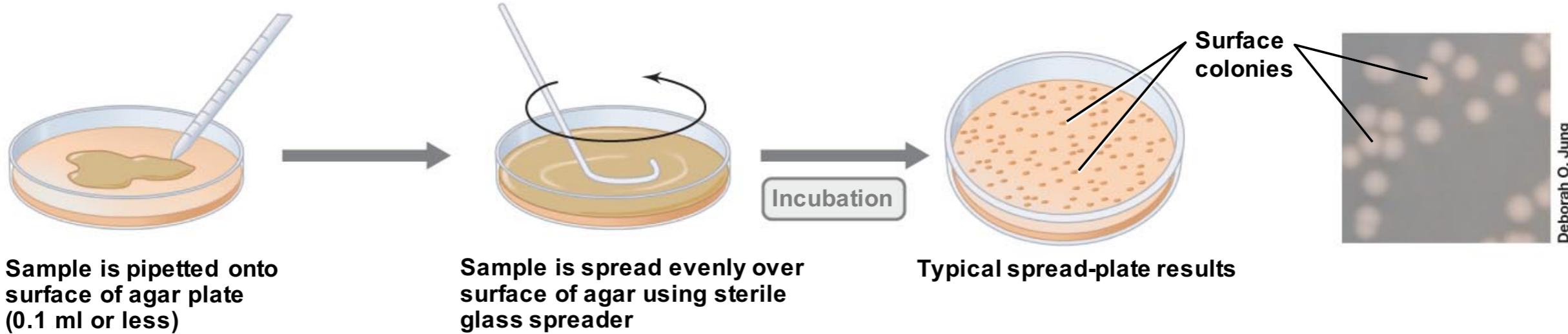
- **Microscopic counts:** Microbial cells are enumerated by microscopic observations (results can be unreliable)
- **Viable counts:** Measurement of living, reproducing population (need to be diluted)
- **Spectrophotometry:** Turbidity measurements are indirect, rapid, and useful methods of measuring microbial growth

Direct microscopic counting procedure using the Petroff-hausser counting chamber

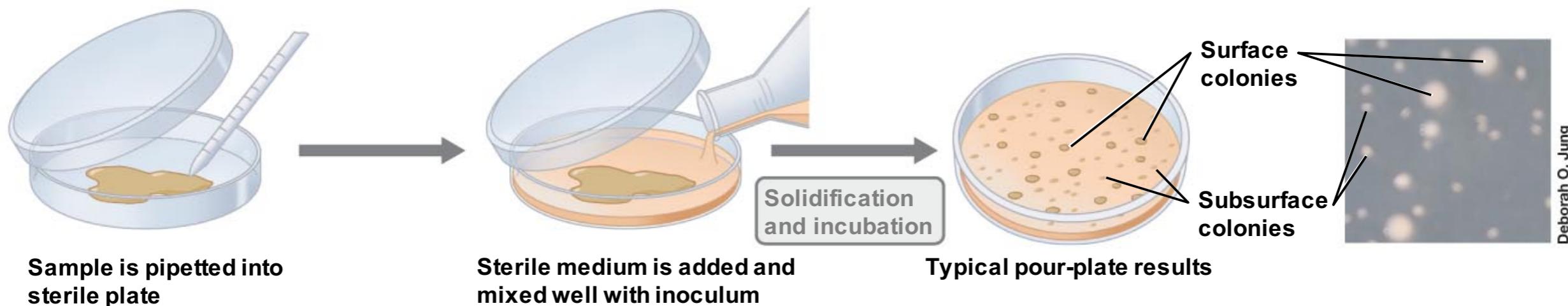


Standard Plate Counts (Viable counting method)

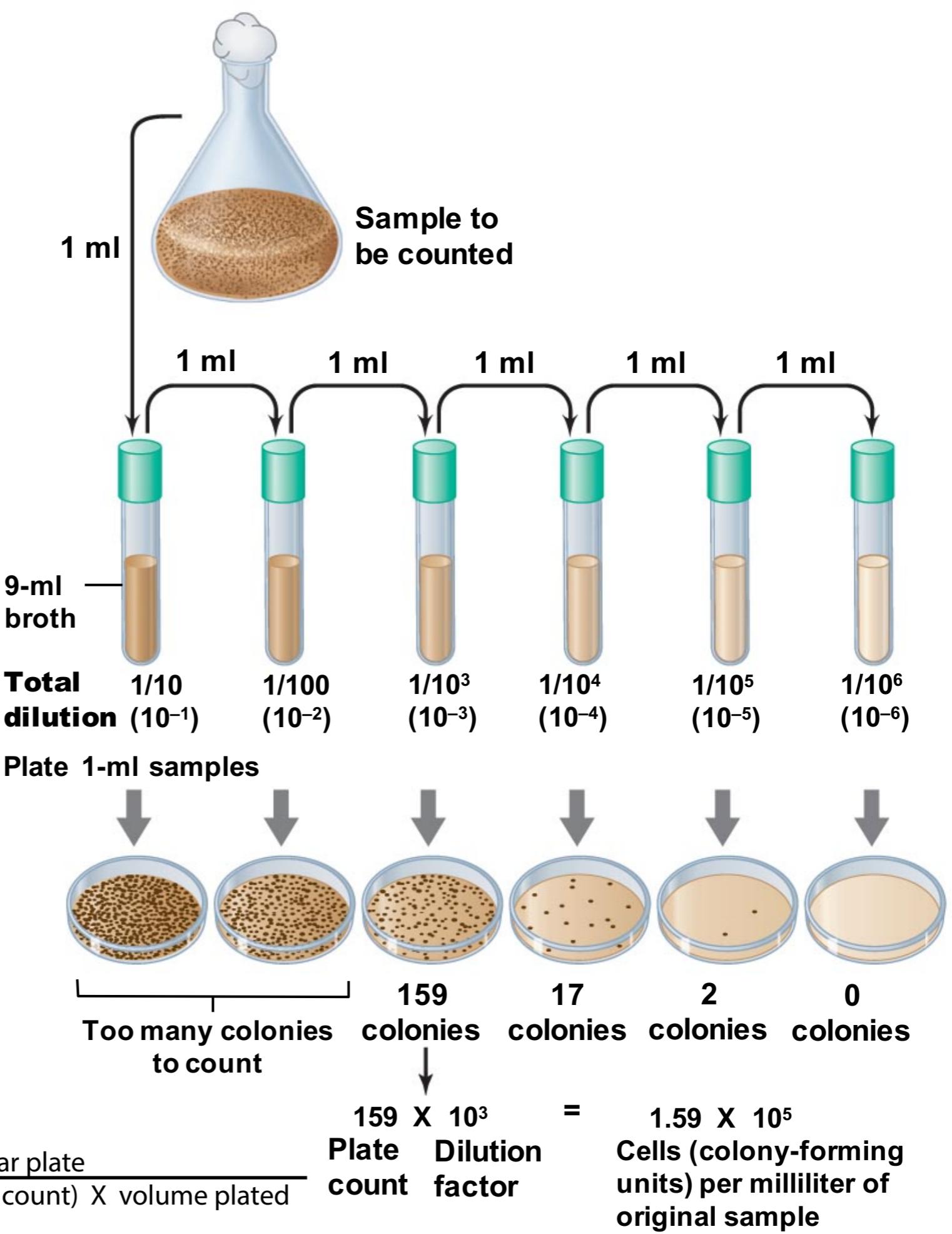
Spread-plate method



Pour-plate method

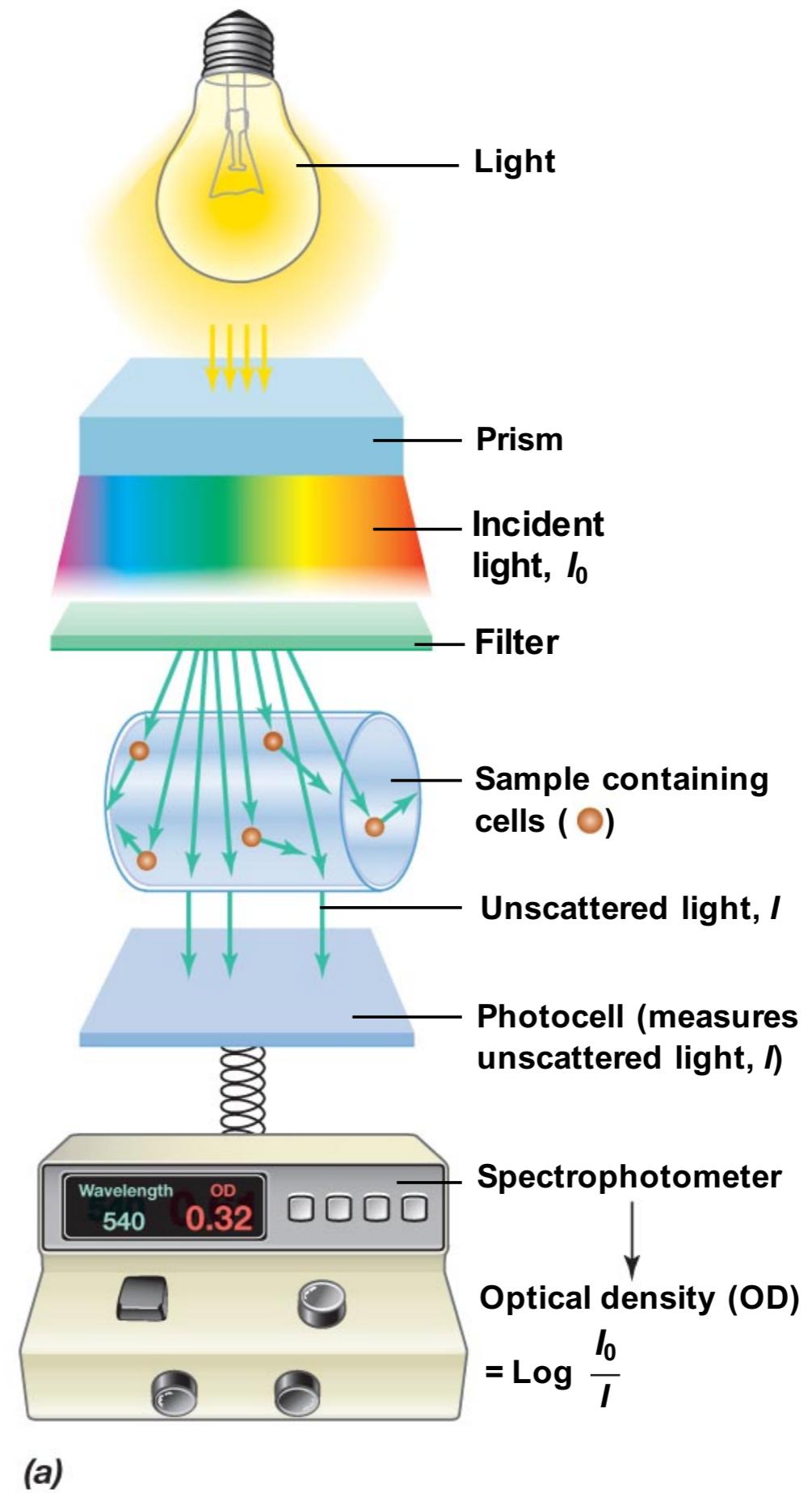
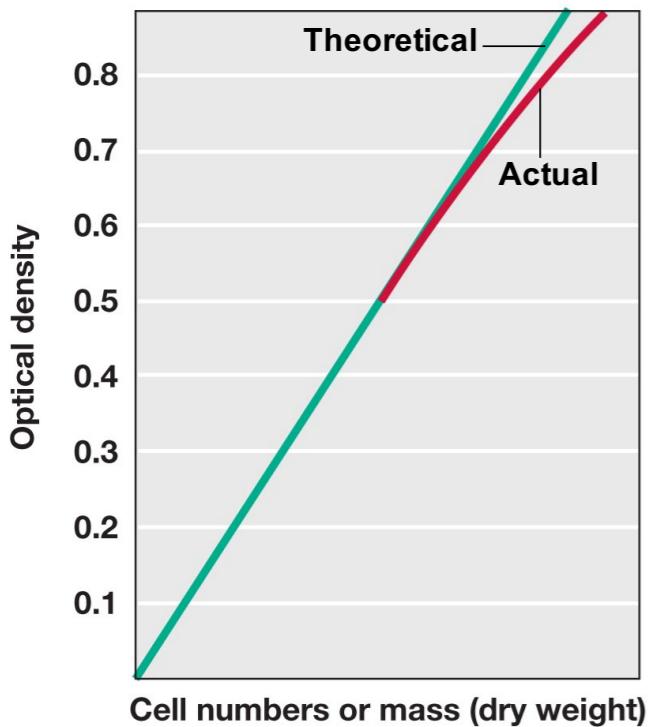


Procedure for viable counting using serial dilutions of the sample and the pour-plate method.



Turbidity measurements of microbial growth

To relate a direct cell count to a turbidity value, a standard curve must first be established



Why do we need to use molecular biological tools?

To avoid the bias of conventional culture-based tools

Habitat	Cultivability (%)
Seawater	0.001 - 0.1
Freshwater	0.25
Mesotrophic lake	0.1 - 1
Unpolluted estuarine waters	0.1 - 3
Activated Sludge	1 - 15
Sediments	0.25
Soils	0.3

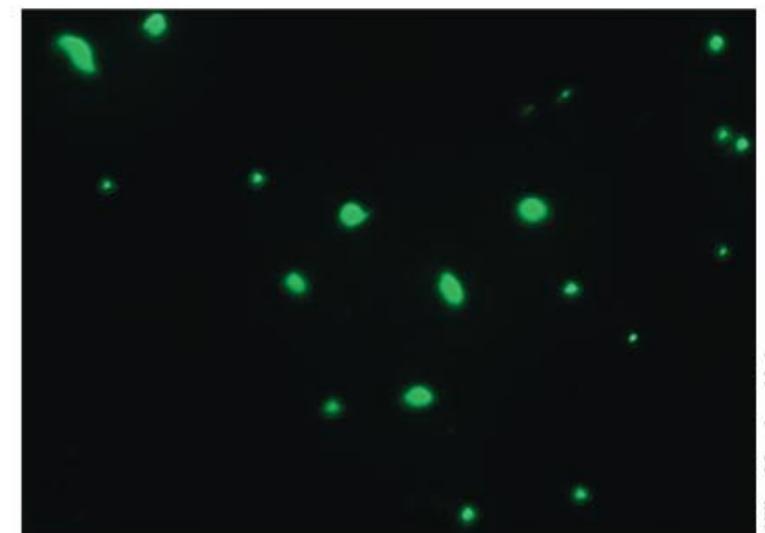
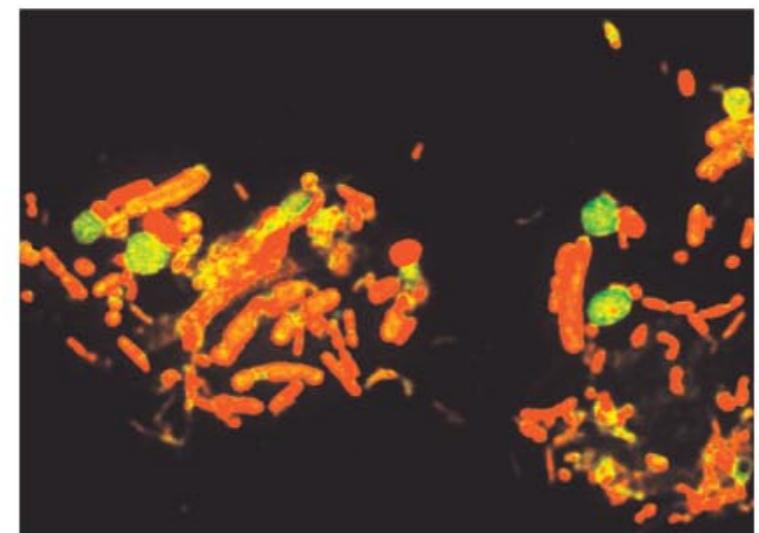
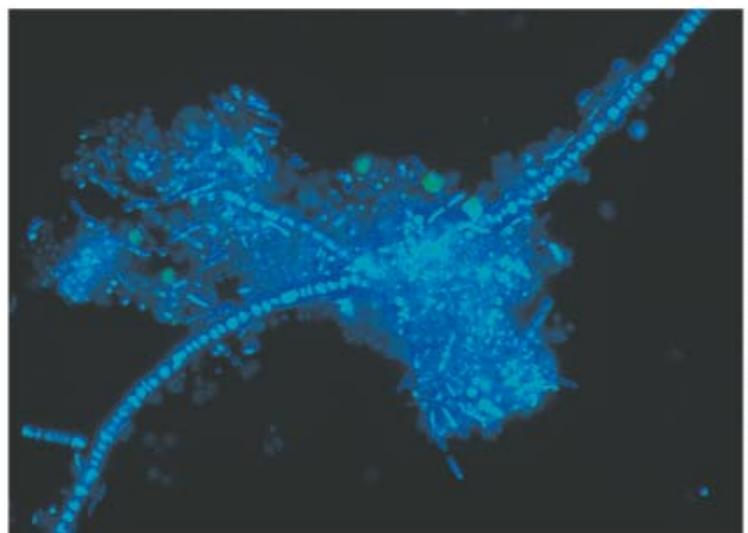
Cultivability = cultivable bacteria / total bacteria (Amann, 1995)

Culture-Independent Microscopic Analyses

- General Staining methods
- Fluorescence In Situ Hybridization (FISH)

General Staining methods

- Fluorescent staining using DAPI, acridine orange (AO), or SYBR Green I (SYBR)
 - DAPI, AO, and SYBR fluoresce under UV light
 - DAPI, AO, and SYBR are used for the enumeration of microorganisms in samples
 - DAPI, AO, and SYBR are **nonspecific and stain nucleic acids**
 - Cannot differentiate between live and dead cells



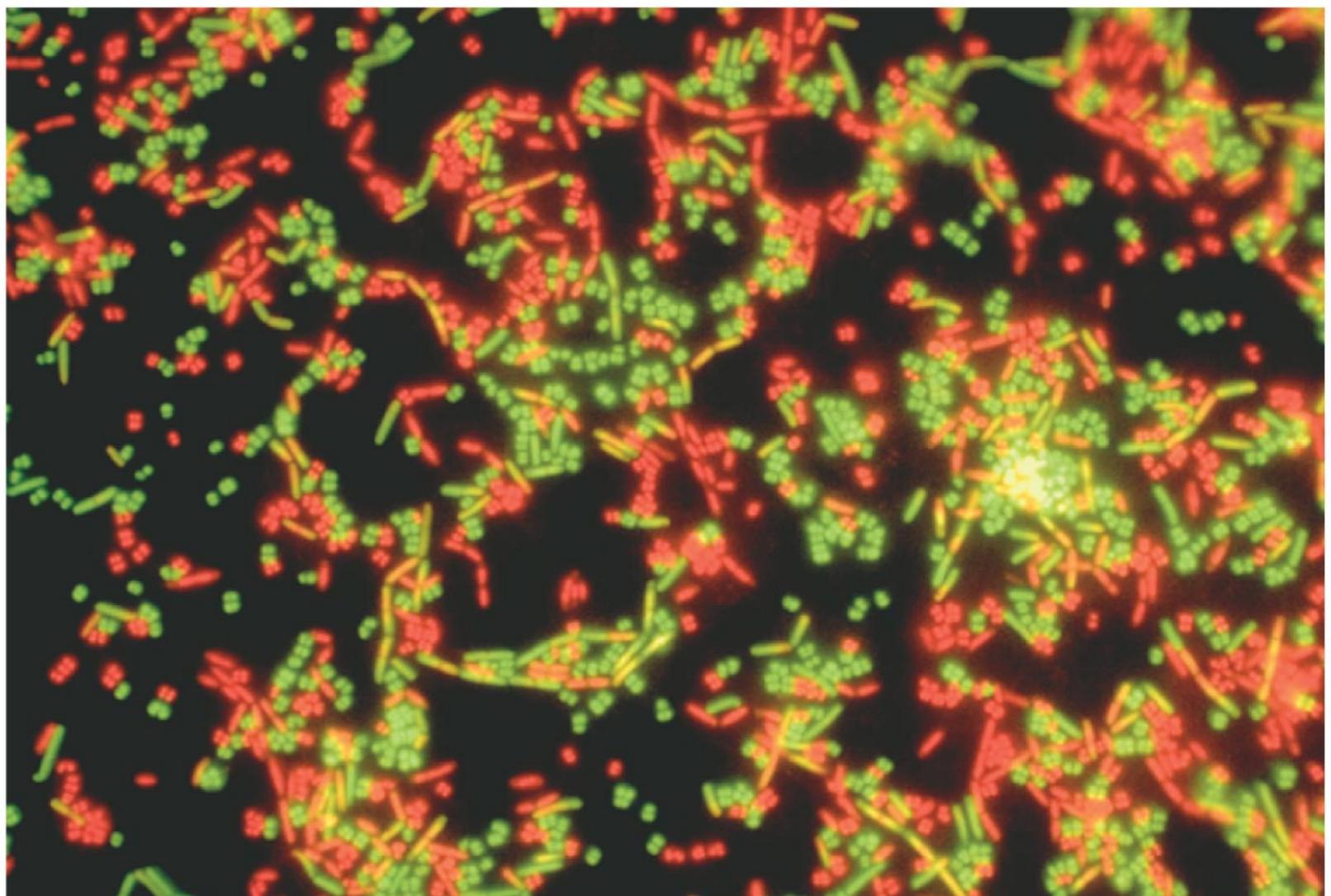
(a) DAPI-stained cells
fluoresce bright blue

(b) AO-stained cells
fluoresce orange or
greenish orange

(c) SYBR-stained cells fluoresce
green

Viability stains: differentiate between live and dead cells two dyes are used

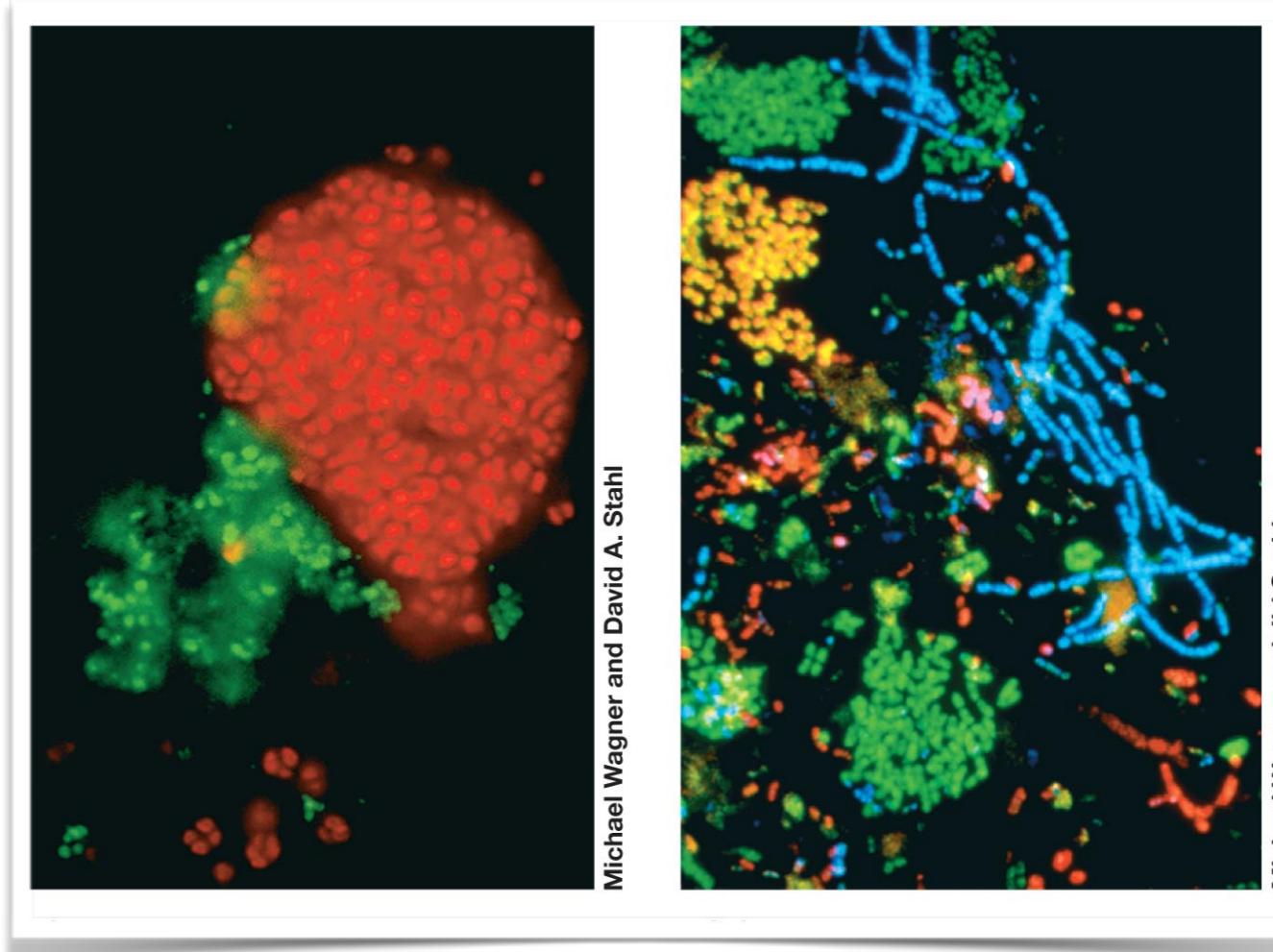
- Based on integrity of cell membrane
- Green cells are live
- Red cells are dead
- Can have issues with nonspecific staining in environmental samples



Molecular Probes, Inc., Eugene, OR

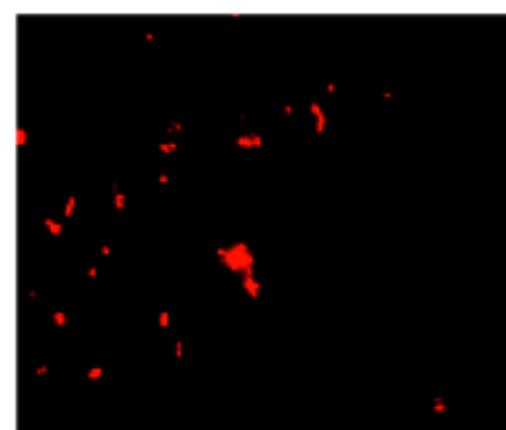
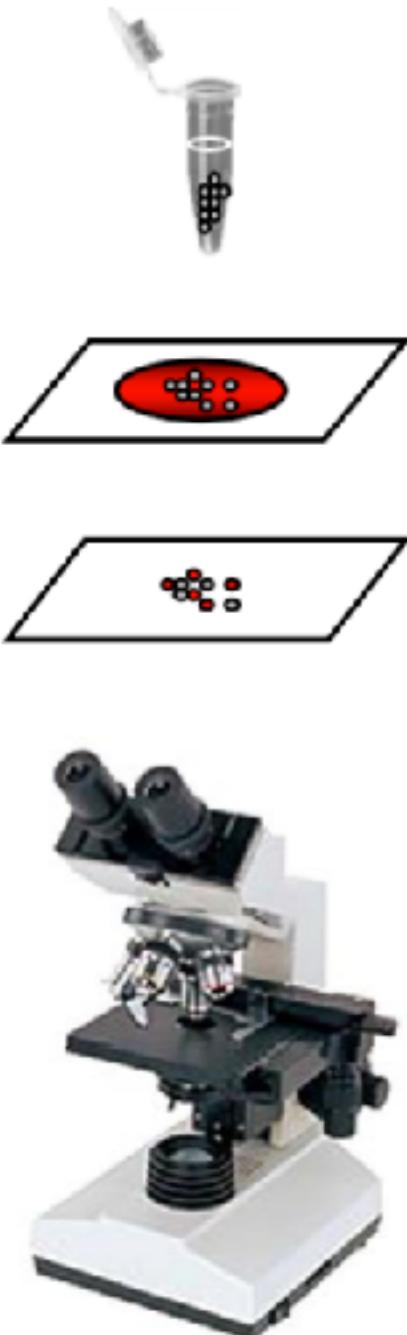
FISH (Fluorescence in situ Hybridization)

- A molecular microbiology technique to detect **DNA or RNA sequences** with fluorescence-labeled probes.
- FISH technology can also employ multiple phylogenetic probes
- Used in microbial ecology, food industry, and clinical diagnostics
- Probe is usually 15 - 30 bases long
- Multi-color probes and autofluorescence

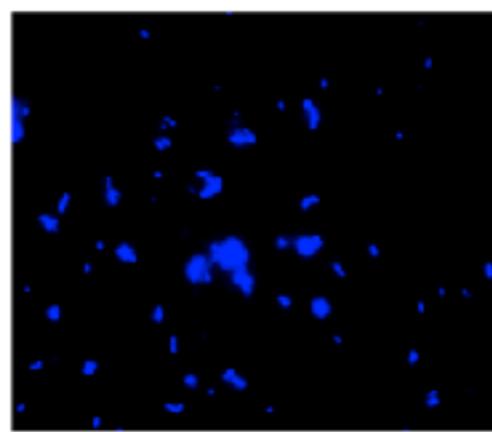


FISH (Fluorescence in situ Hybridization)

- Basic step
 1. Fix cells to make cell membrane permeable to probes (PFA/ethanol)
 2. **Hybridize with fluorescence-labeled probes**
 3. Wash off unbound probes
 4. Stain all cells with DAPI
 5. Visualize under a fluorescence microscope

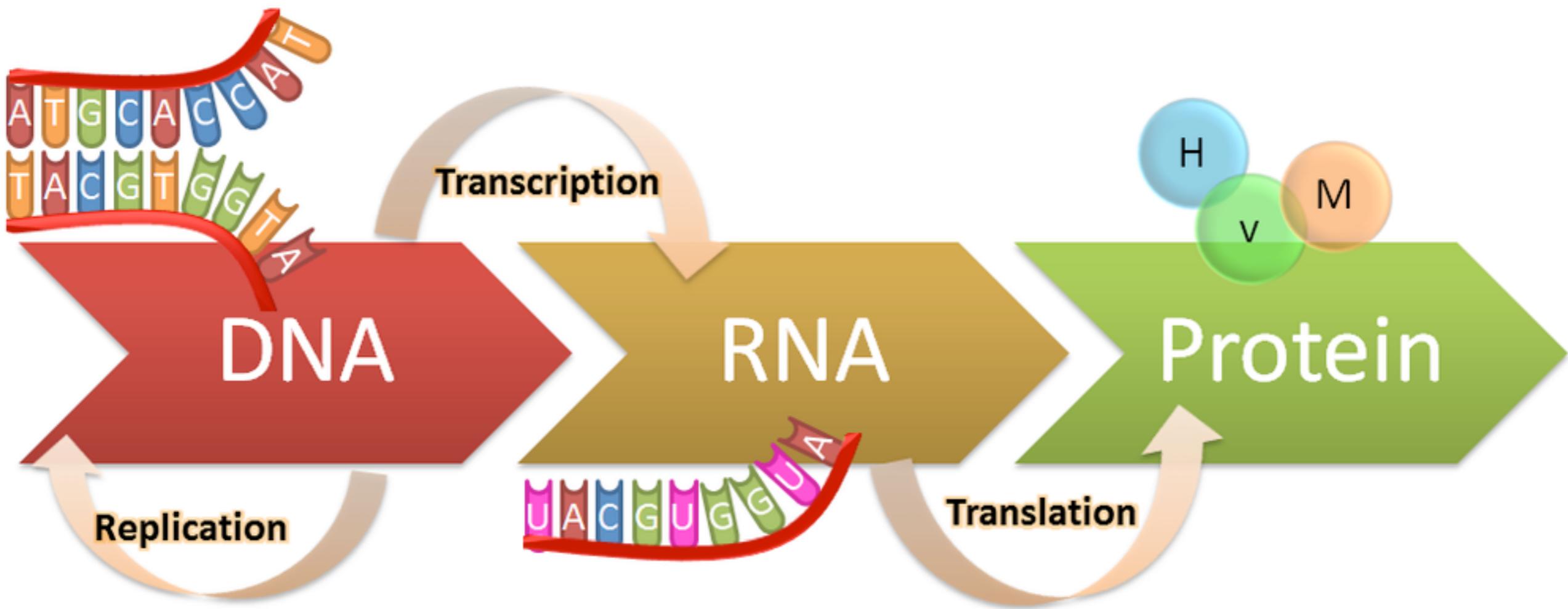


Hybridized with a phylogenetic probe



DAPI-stained cells

The central Dogma of Molecular biology

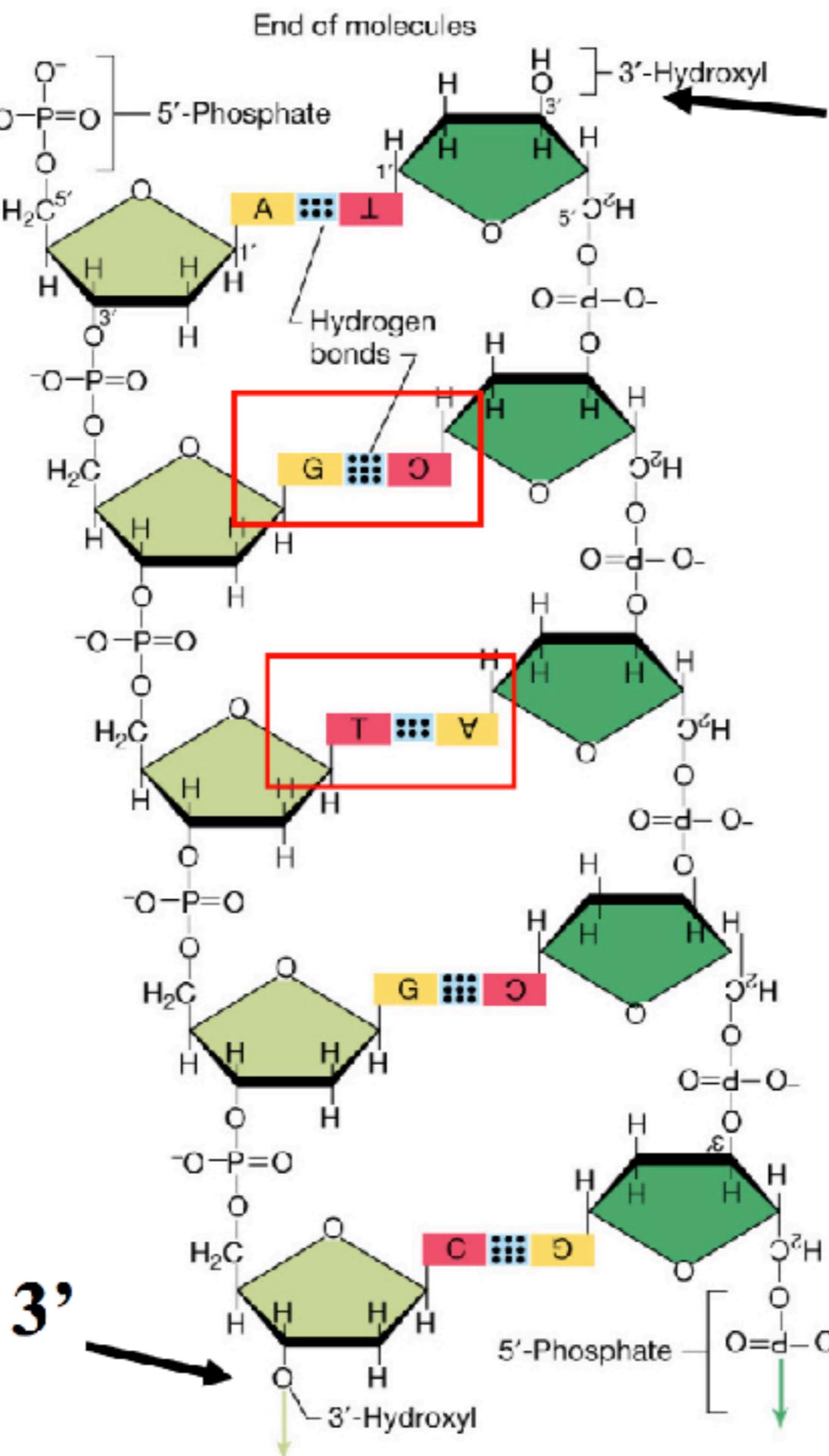


Three informational macromolecules in cell

DNA : **genetic blueprint for the cell**

RNA: **intermediary molecule that converts this blueprint into defined amino acid**

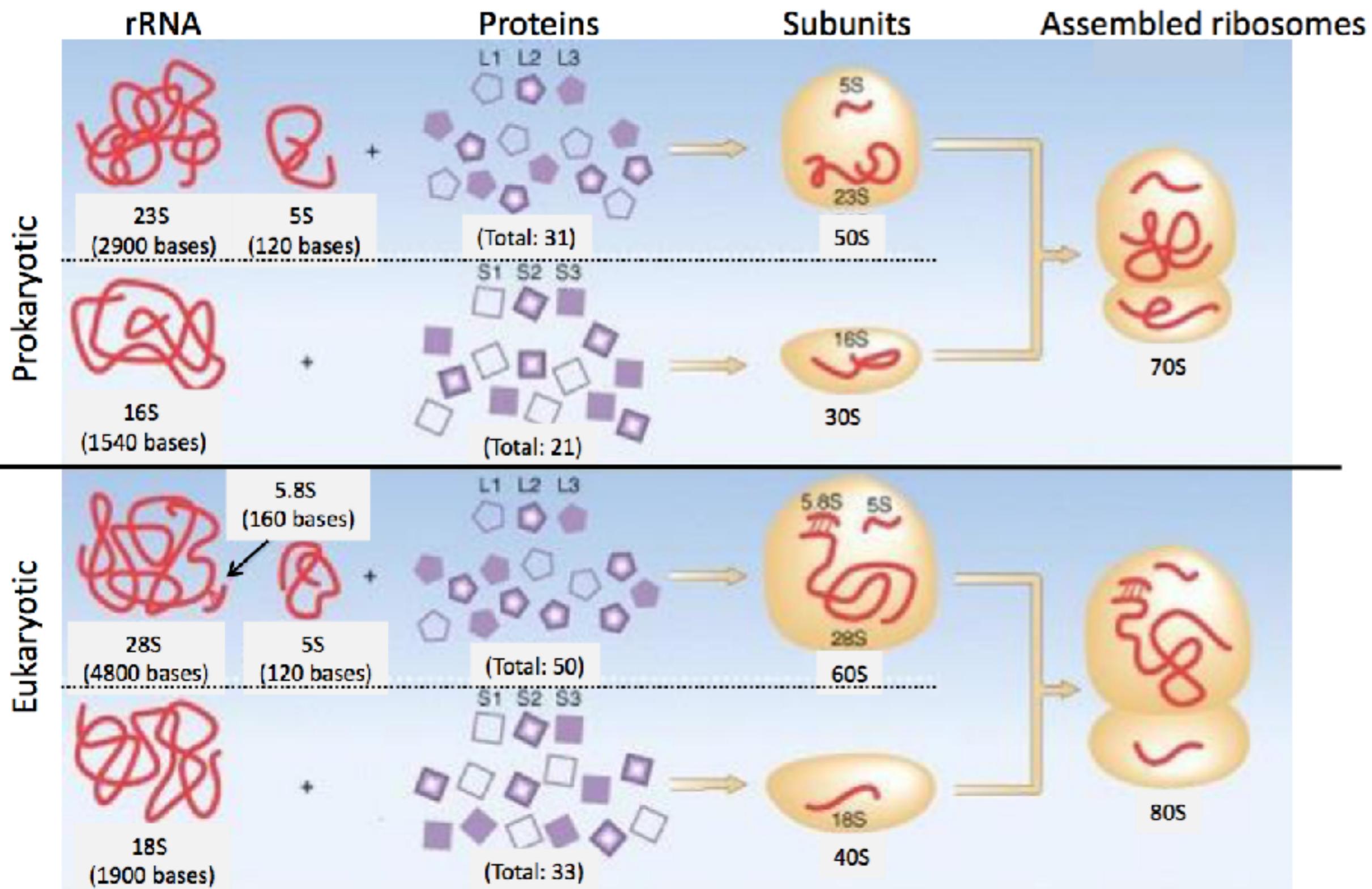
Protein: **amino acid sequence (polypeptide)**

DNA **5'** →**DNA elongated at 3' ends**

Molecule has direction
&
Strands run
anti-parallel

Figure 4.4

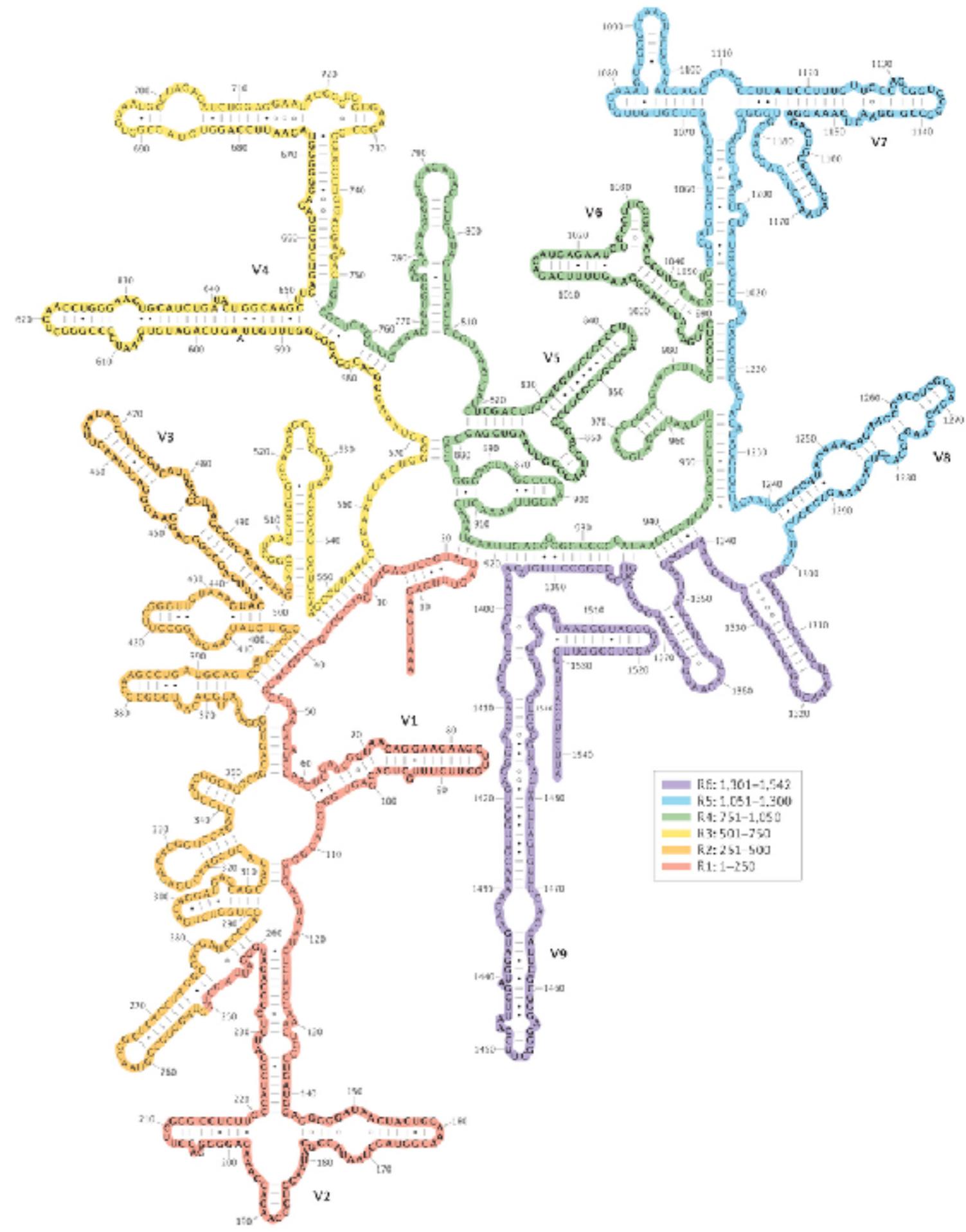
Composition of Ribosomes



Source: <http://www.tutorvista.com/content/biology/biology-iii/cell-organization/nonmembranous-cell-organelles.php>

16S rRNA

- Proper target size
- Showing evolution of history
- Highly conserved between different species of bacteria and archaea.



Exercise

The following 16 rRNA genes from three organisms in an aeration tank have been sequenced. Design a specific phylogenetic probe to target organisms A and B. (Hints: What's the common sequence of these organisms? What's the direction of the probe?)

Organism A: 5' caccagtagc gcgtgttcat ggaaggcgcc tgcgaggctg tcgagcgggc aggcatcgac
atgcgcaccc tgcgcggtgg acgcaccggc gtcttcgccc gcgtgatgta ccacgactac 3'

Organism B: 5' ccccagcaga acgtgttcct cgagacggtg tgggagacct tcgaatccgc cggagtggac
ccgcgcgcgg tacgcggtcg ttccgtcgaa gtcttcgccc gcgtgatgta acaggactac 3'

Organism C: 5' ctccagtagc tcgtgttcat cgaaggcgcc ttgcgaggctg tcgagggggc aggcatggac
atgcgaaccc tgcgtgggtgg acgctccggc gtcttccccg gagtgatgaa ccacgactac 3'

Answer

The following 16 rRNA genes from three organisms in an aeration tank have been sequenced. Design a specific phylogenetic probe to target organisms A and B.

Organism A:	5' caccagtagc	ggtgttcat	ggaagcgcc	tgcgaggctg	tgcggggc	aggcatcgac
	atgcgcaccc	tgcgggtgg	acgcacccggc	gttttcgacg	ggtgtatgt a	ccacgactac 3'
Organism B:	5' ccccagcaga	acgtgttct	cggacggtg	tgggagacct	tgcataccgc	cggagtggac
	ccgcgcgcgg	tacgggtgg	ttccgtcgaa	gttttcgacg	ggtgtatgt a	acaggactac 3'
Organism C:	5' ctccagtagc	tctgttcat	cgaagcgcc	tgcgaggctg	tgcggggc	aggcatggac
	atgcgaaccc	tgcgtgggtgg	acgctccggc	gtttcccccg	gagtatgaa	ccacgactac 3'
				↑	↑	
			target: 5'	gttttcgacg	ggtgtatgt 3'	
			probe: 3'	cagaagcgcc	cgcactacat 5'	

- Identify the common sequence
- Identify reverse (5' to 3' → 3' to 5') complement (base pairing) sequence of the common sequence

Culture-Independent Genetic Analyses

- PCR methods of Microbial Community Analysis
- Microarrays for Analysis of Microbial Phylogenetic and Functional diversity
- Environmental Genomics and Related Methods

BLAST

BLAST®

Home Recent Results Saved Strategies +

Basic Local Alignment Search Tool

BLAST finds regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance.

[Learn more](#)

NEWS

The BLAST widget - integrating your BLAST results into NCBI's Genome Data Viewer!

Tue, 31 Jul 2018 18:00:00 EST

[More BLAST news...](#)

Web BLAST

Nucleotide BLAST
nucleotide ► nucleotide

blastx
translated nucleotide ► protein

tblastn
protein ► translated nucleotide

Protein BLAST
protein ► protein

ATACAGAGGGTCAAGCGTTAACGAAATTACTGGCGTAAAGCGTACGT
AGGTGGCTGAATAAGTCAGATGTGAAATCCTCGAGCTCAACTTGAGAATT
GCATTGAAACTGCTTAGCTAGAGTATGGTAGAGGGAAGTGGATTCCAC
GTGTAGCGGTAAATGCGTAGATATGTGGAGGAACATCAGTGGCGAAGGC
GACTTCCTGGACTAATACTGACACTGAGGTACGAAAGCGTGGGAGCAA
CAGGATTAGATACCTGGTAGTCCACGCCCTAACGATGAGAACTAGATG
TTGGGGAGTTAACGCTTACTATCGCAGCTAACGCGTTAACGTTCTCCG
CCTGGGGAGTACGCCGCAAGGTTAAAACCAAAGGAATTGGCGGGG

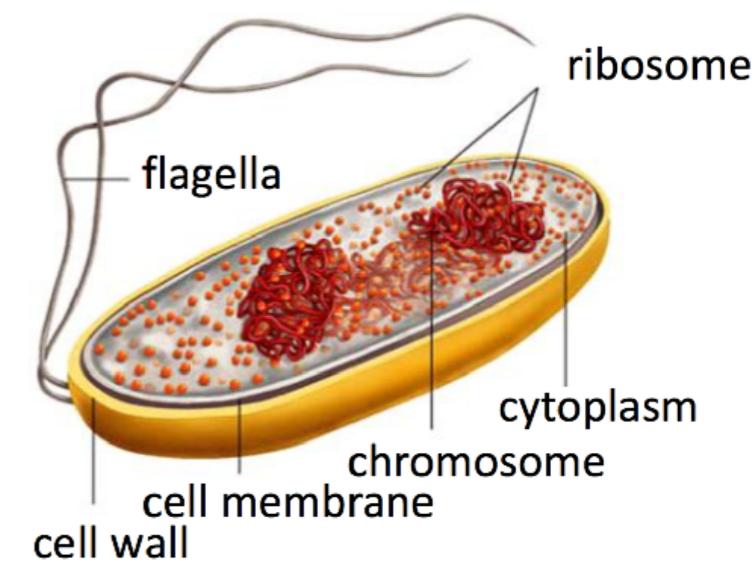
TAAGCCTCTGTTAAAGCTCGGGCCAACCCGGAAAGCGCAGAGGGTAC
TGCCAGGCTAGAGGGGGAGAGGGAGCGCGGAATTCCGGTGTAGCGGTG
AAATGCGTAGAGATCGGGAGGAAGGCCGTGGCGAAGGCCGCTCTGGA
ACCTACCTGACGCTGAGACACGAAAGCGTGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCCTAAACTATGGATACTAGGTGTCGGCGGGTTA
CCGCCGGGCCAGCTAACGCTAACGCTAACGCTAACGCTAACGCTAACG
CGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCGACAAGCGGTGGA
GCATGTGGTTAACGCAAGCAACGCGAAGAACCTTACCCAGGTTGGACA
TGCACGTAGTAGAAGGGTAAAGCCTAACGAGGTAGCAATACCAGCGTGC
TCAGGTGCTGCATGGCTGTCAGCTCGTGCCGTGAGGTGTTGGTTAA
GTCCCGCAACGA

PCR Methods of Microbial community analysis

- Specific genes can be used as a measure of diversity
 - Techniques used in molecular biodiversity studies
 - DNA isolation and sequencing
 - PCR
 - Restriction enzyme digestion
 - Electrophoresis
 - Molecular Cloning

Nucleic Acid Extraction Kits

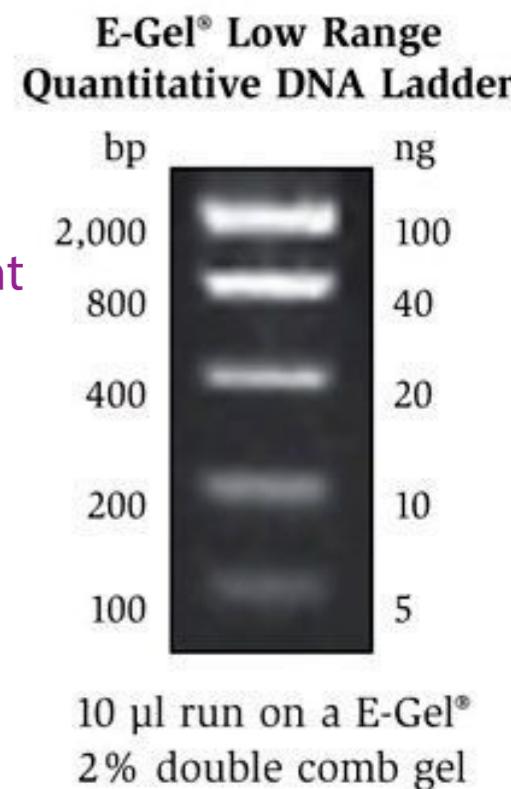
- Components are typically proprietary
- General steps
 1. Lyse cells
 2. Remove debris
 2. Precipitate proteins
 3. Bind target nucleic acid to column matrix
 4. Wash nucleic acid
 5. Elute nucleic acid
 - DNase or RNase treatment if necessary



DNA concentration and Purification

- Concentration and purity
 - Commonly assessed through absorbance
- Concentration: A260nm
 - Absorbance of 1.0 (1-cm path length) = 50 µg DNA/mL
- Purity: A260nm/A280nm
 - High quality DNA generally gives ratio of **1.7-2.0**, with **1.8 often cited as “best”**
 - Each nucleotide base has a different A260nm/A280nm ratio, so that is why a range is listed for high-quality DNA
- Lower ratios (<1.7) suggest **protein contamination**

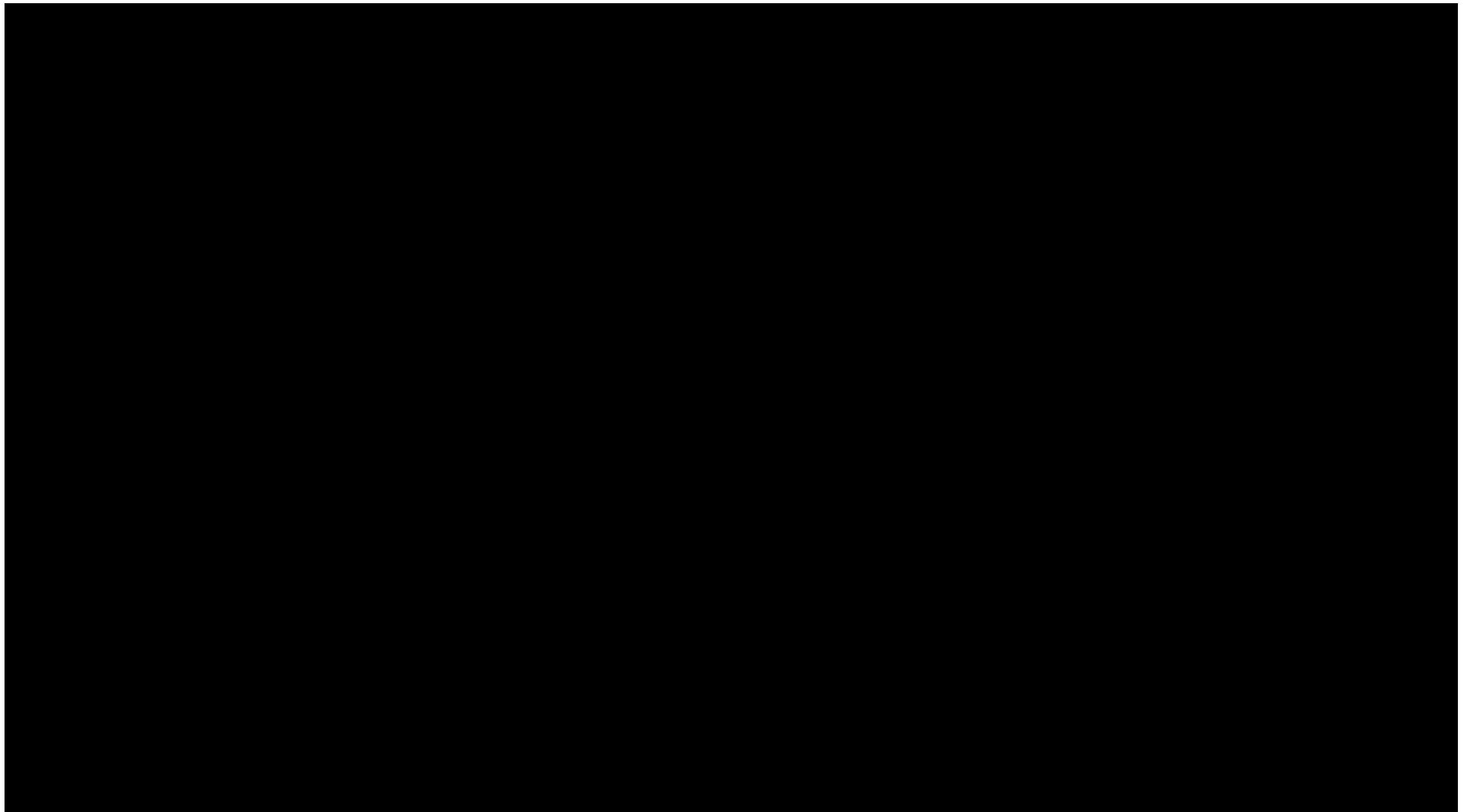
Also use mass DNA ladders (gel) or fluorescent DNA-binding dyes



Polymerase Chain Reaction (PCR)

- PCR = **Amplification of Target DNA sequence**
- Reaction Contains
 1. DNA targets
 2. **2 oligonucleotide primers: 17-30 bases, ea**
one primer binds to each stand

16S primers: 8f (5'-AGAGTTGATCCTGGCTCAG-3')
1492r (5'-GGTTACCTTGTACGACTT-3')
 3. Mixtures of dNTPS: Building blocks of DNA
 4. Taq polymerase: Synthesizes new DNA
 5. Buffer: appropriate salt Conc.

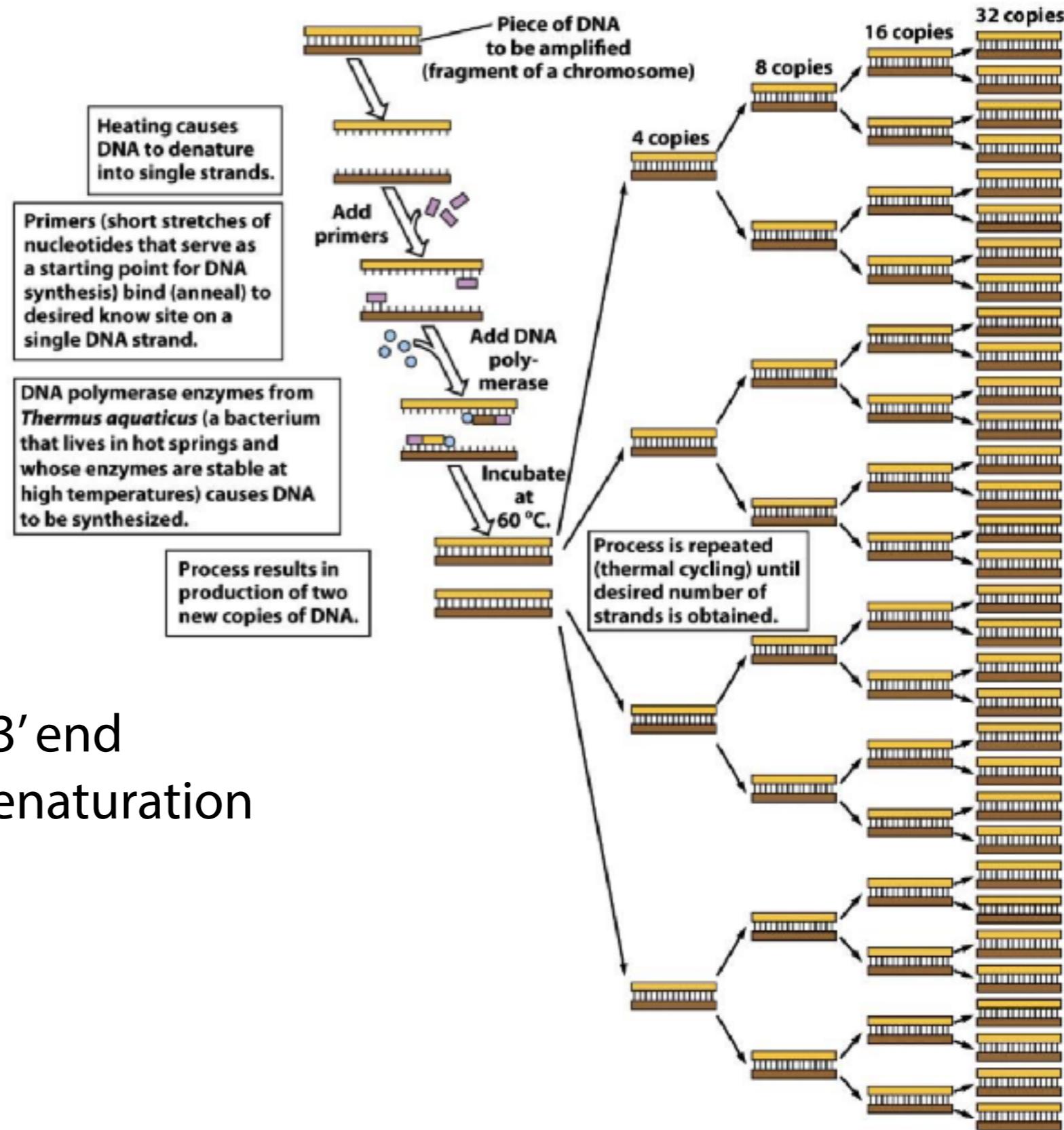


PCR Songs

Polymerase Chain Reaction (PCR)

Basic steps

- 1) Denaturation: 95°C
 - to separate DNA strands
- 2) Annealing : 45- 60°C
 - Primer binding
- 3) Extension : 72°C
 - ~ 10000 bases /min
 - To synthesize new DNA at 3' end
 - Taq polymerase survives denaturation



Real-time PCR

- More reliable relationship between quantity of target DNA in original sample and number of PCR cycles to produce a detectable amplicon
 - CT: number of cycles to produce detectable amplicon (or cycle threshold)
 - Convenient to detect the amplicon as it is produced. How?
 - Nonspecific fluorescent probes (e.g., Sybr Green)
 - Fluoresces when bound to double stranded DNA
 - Specific fluorescent probes (e.g., TaqMan®-probes)
 - Increase in sample fluorescence as specific product is synthesized
- Higher quantities of target DNA in original sample yield lower CT values.

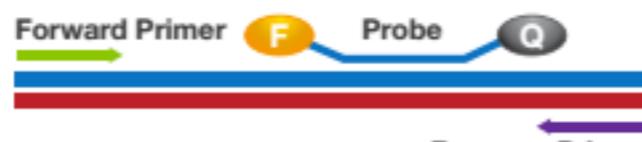
Real-time PCR

Real-Time PCR allow for the detection of PCR amplification during the early phases of the reaction.

QPCR Reaction Detail

Step 1:

Primers and probe bind to target DNA.



Step 2:

PCR occurs, primers are extended on forward and reverse DNA strands.



Step 3:

Probe is degraded as a result of polymerization and fluorescent signal is generated.

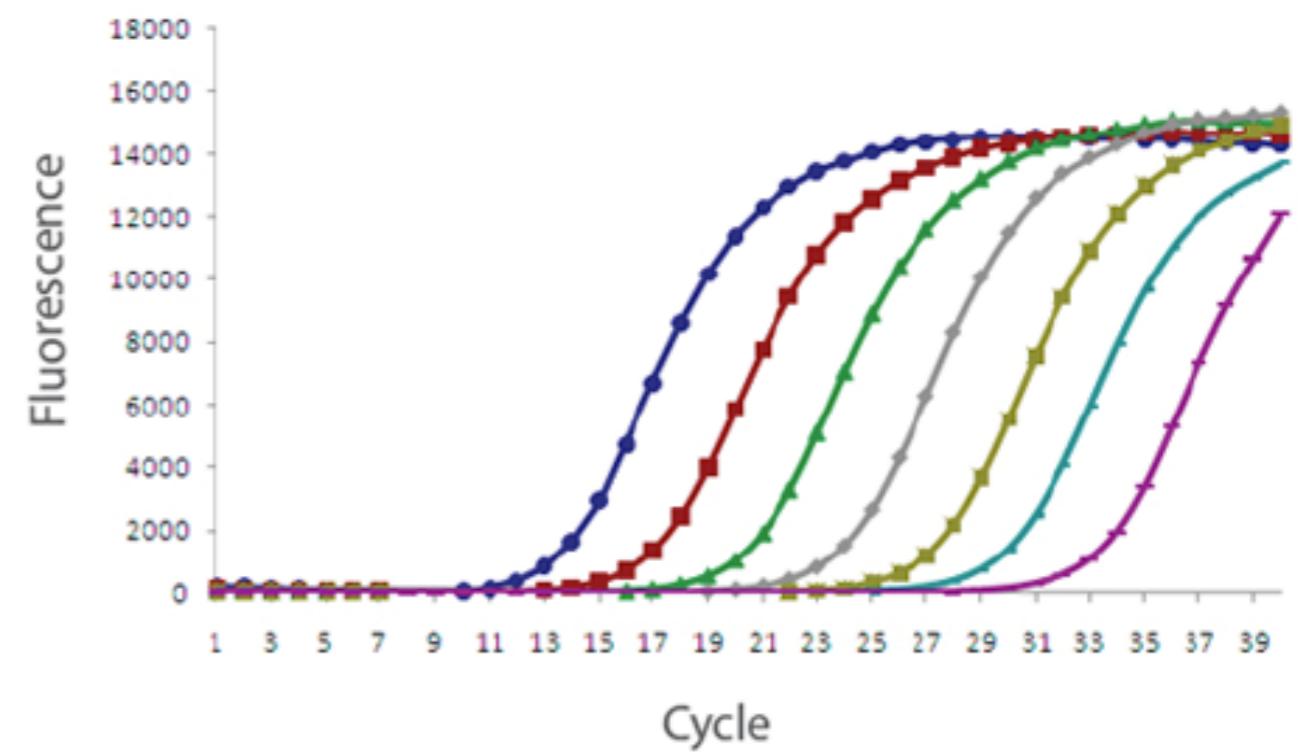


Step 4:

Target DNA is amplified and fluorescent signal can be measured and quantified.



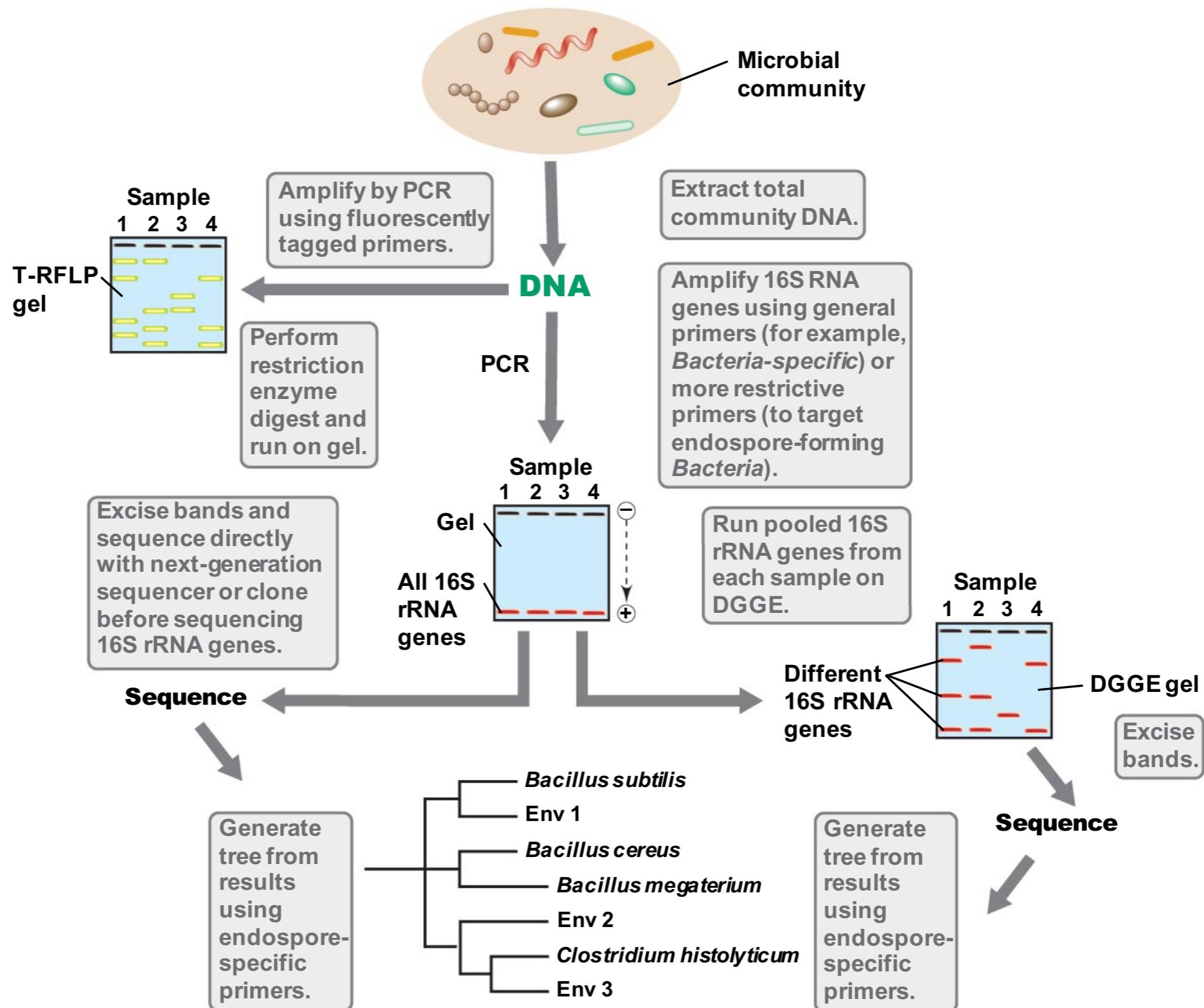
qPCR amplification plot with VisiBlue™



Data analysis and interpretation

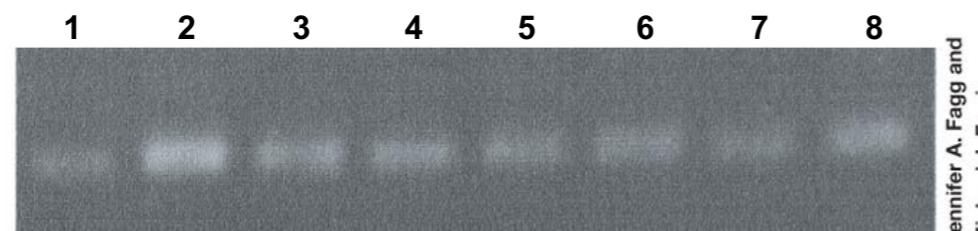
- Absolute quantification
 - Comparison of unknown samples to an external standard provides an accurate and reliable method for the quantitation of nucleic acids
- Normalization: mass of total DNA or volume of water
- Data presentation: mean and standard deviation
- Statistical analysis
 - Parametric and nonparametric methods

Steps in single-gene biodiversity analysis of a microbial community

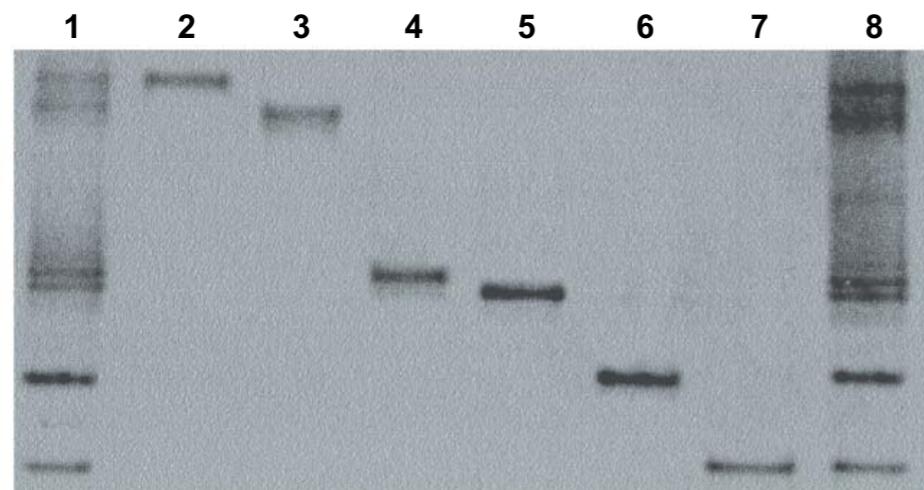


DGGE

- DGGE: denaturing gradient gel electrophoresis separates genes of the same size based on differences in base sequence
 - Denaturant is a mixture of urea and formamide
 - Strands melt at different denaturant concentrations

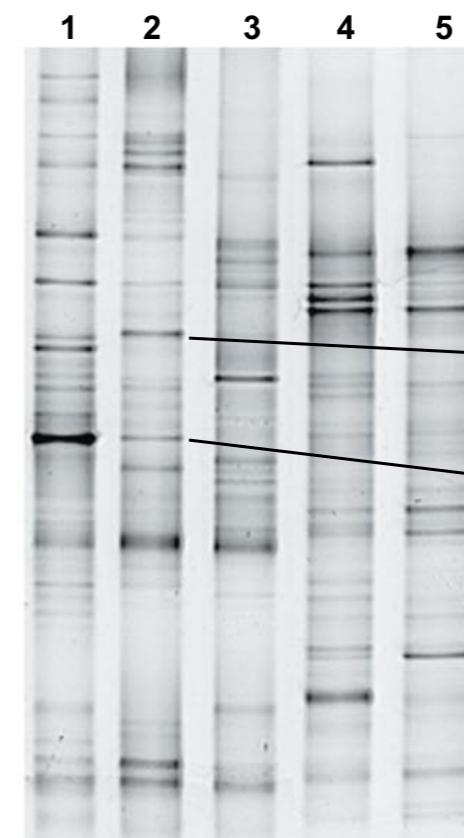


(a) PCR amplification



(b) DGGE

Jennifer A. Fagg and Michael J. Ferris



(c) DGGE of wastewater treatment plants

This phylotype appears to be universal.

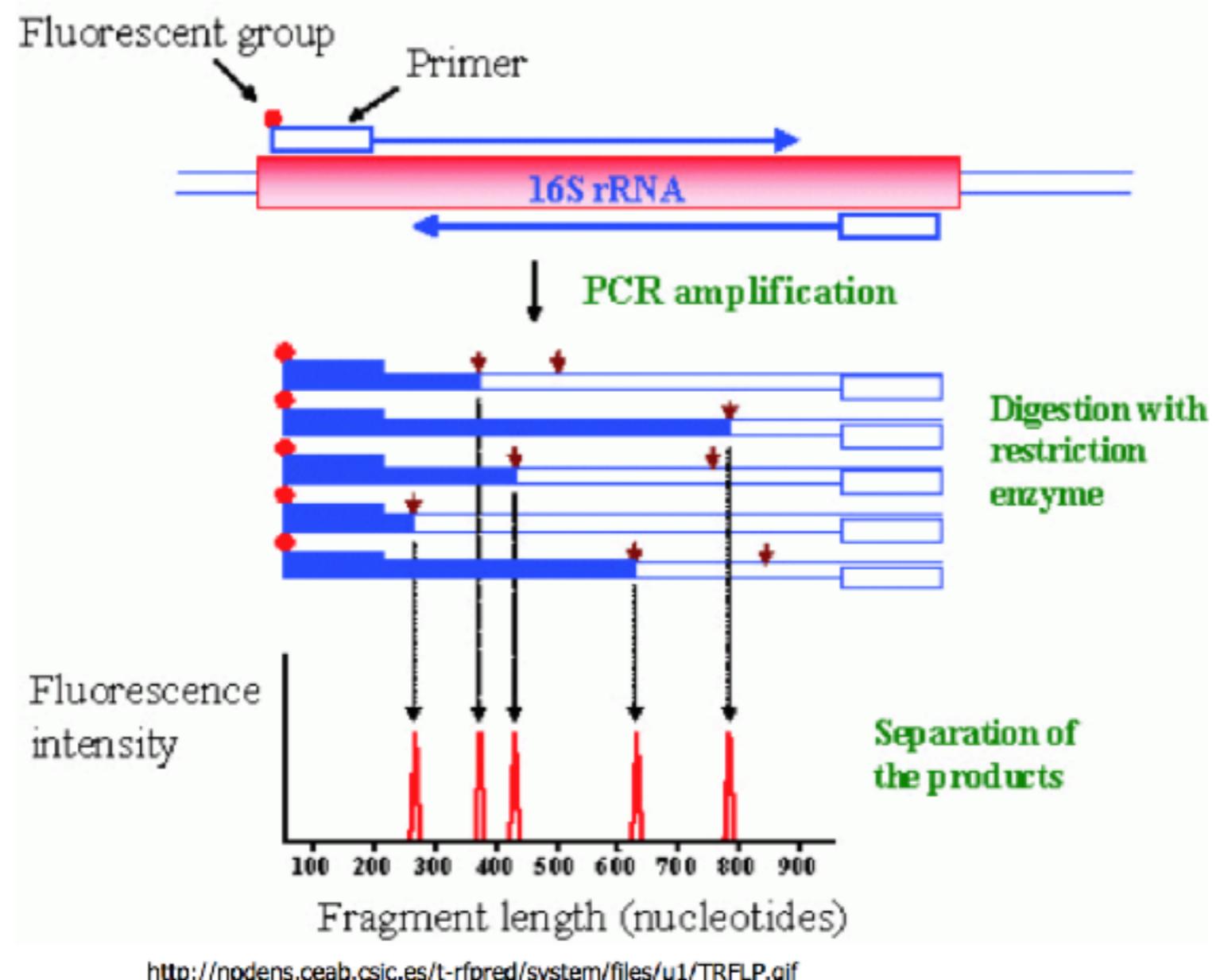
This phylotype is unique to treatment facility 2.

This phylotype is present in both treatment facilities 1 and 2.

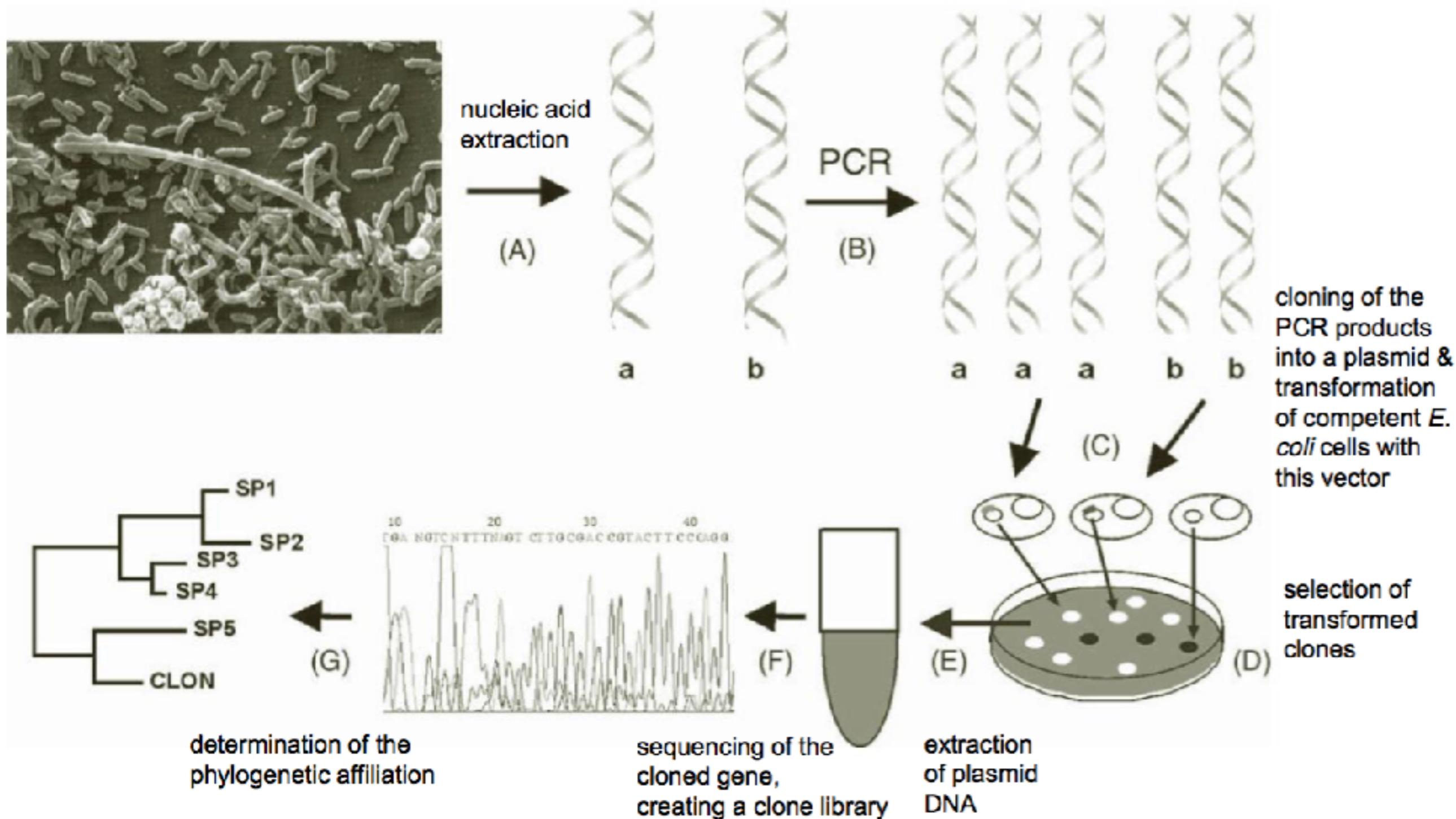
Gerard Muyzer and Abdelaziz Bellai

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Target 16S rRNA sequence is amplified with PCR, and the products are digested with restriction endonuclease, separated by gel electrophoresis, and detected on an automated sequence analyzer.



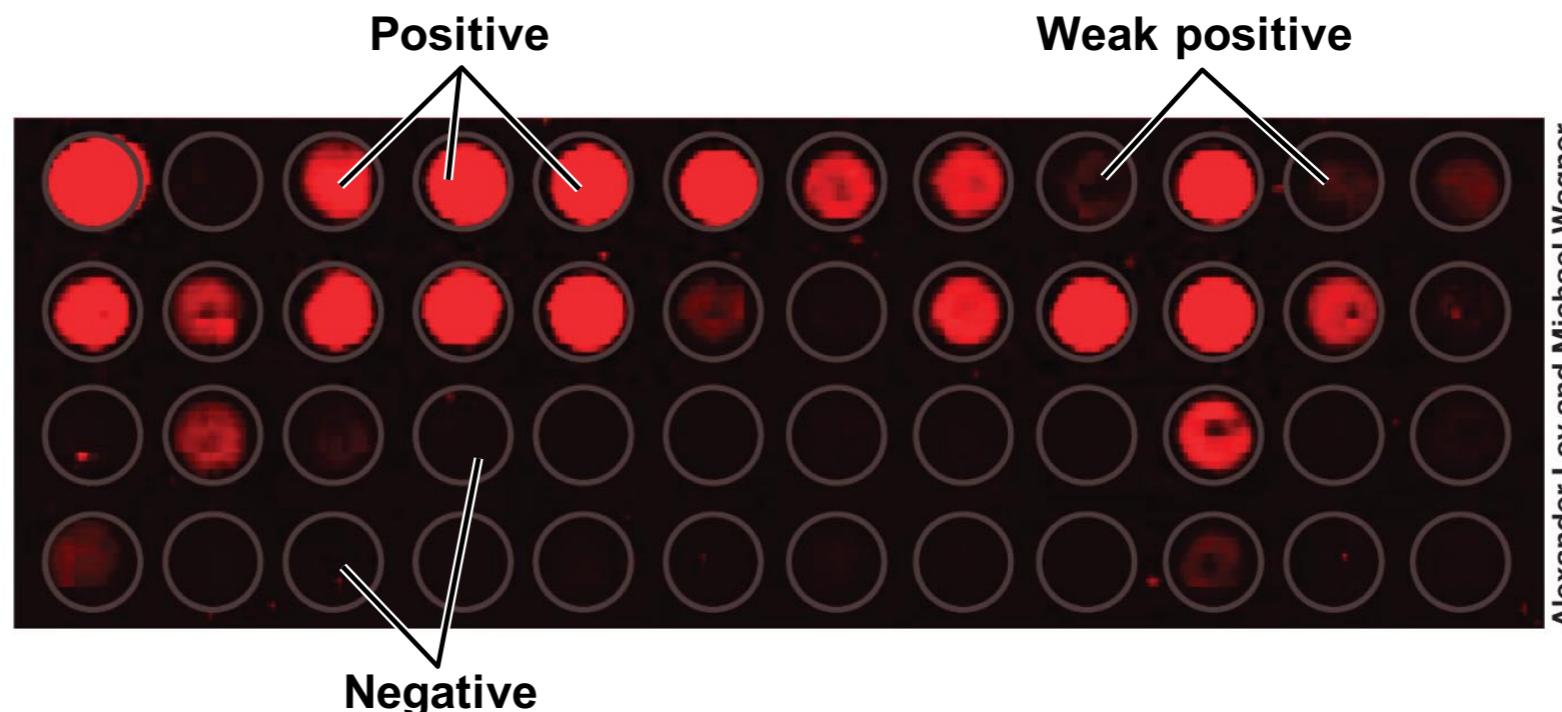
Cloning and Sequencing



Source: Jose L Sanz and Thorsten Kochling, Molecular biology techniques used in wastewater treatment: an overview, Process Biochemistry 42 (2007) 119-133

Microarray

- Phylochip: microarray that focuses on phylogenetic members of microbial community
 - Circumvents time-consuming steps of DGGE and T-RFLP
- Functional gene microarray: microarray that focuses on genes of biochemical significance
 - Encompasses many different metabolic pathways



Alexander Loy and Michael Wagner

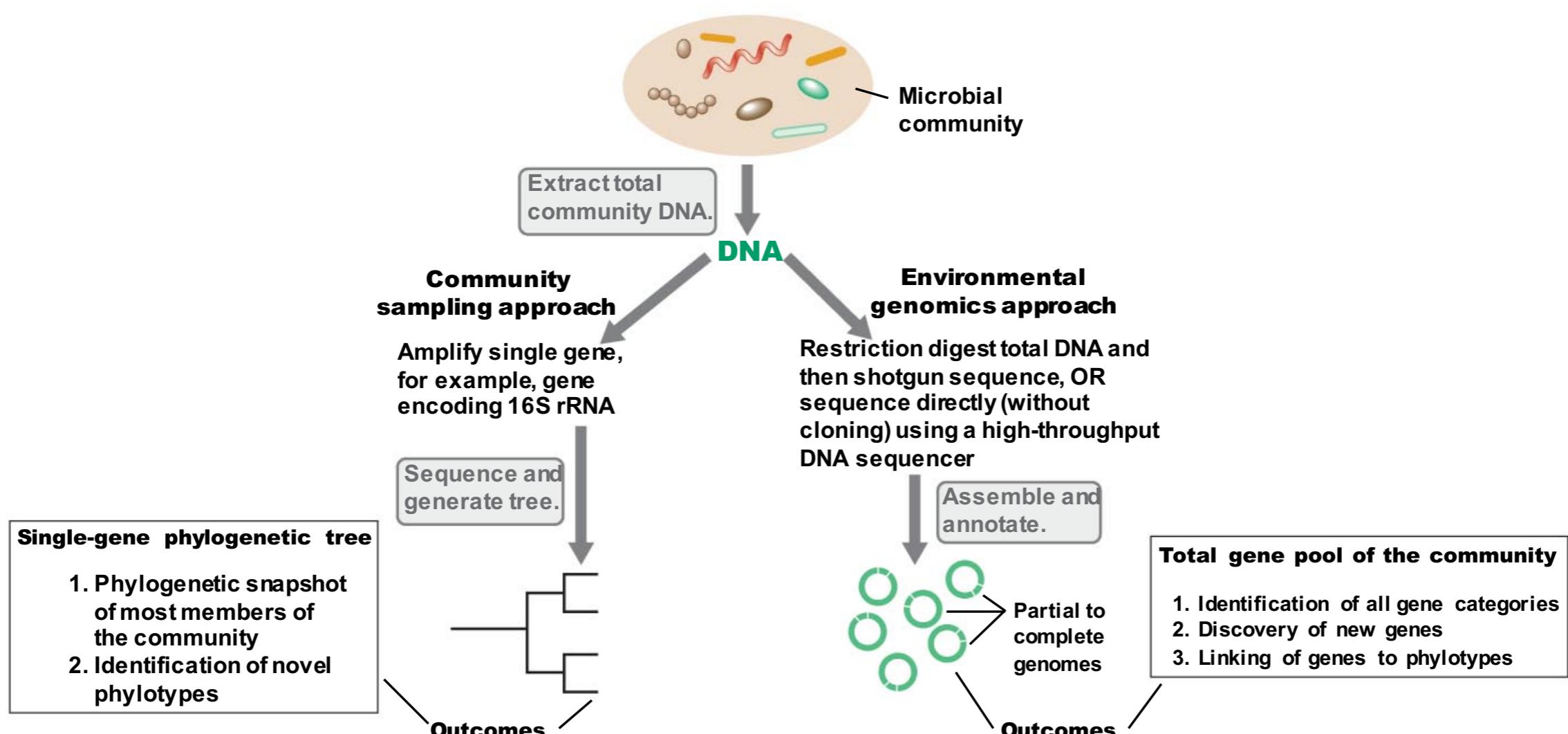
Phylochip analysis of sulfate-reducing bacteria diversity.

Next-generation Sequencing

- Next-generation DNA sequencers do not require a cloning step
- PCR products can be used directly for sequencing
- Allows for the detection of minor phylotypes
- Results of PCR phylogenetic analyses
 - Several phylogenetically distinct prokaryotes are present
 - rRNA sequences differ from those of all known laboratory cultures
 - Molecular methods conclude that fewer than 0.1% of bacteria have been cultured

Environmental Genomics

- DNA is cloned from microbial community and sequenced
- Detects as many genes as possible
- Yields picture of gene pool in environment
- Can detect genes that are not amplified by current PCR primers
- Powerful tool for assessing the phylogenetic and metabolic diversity of an environment



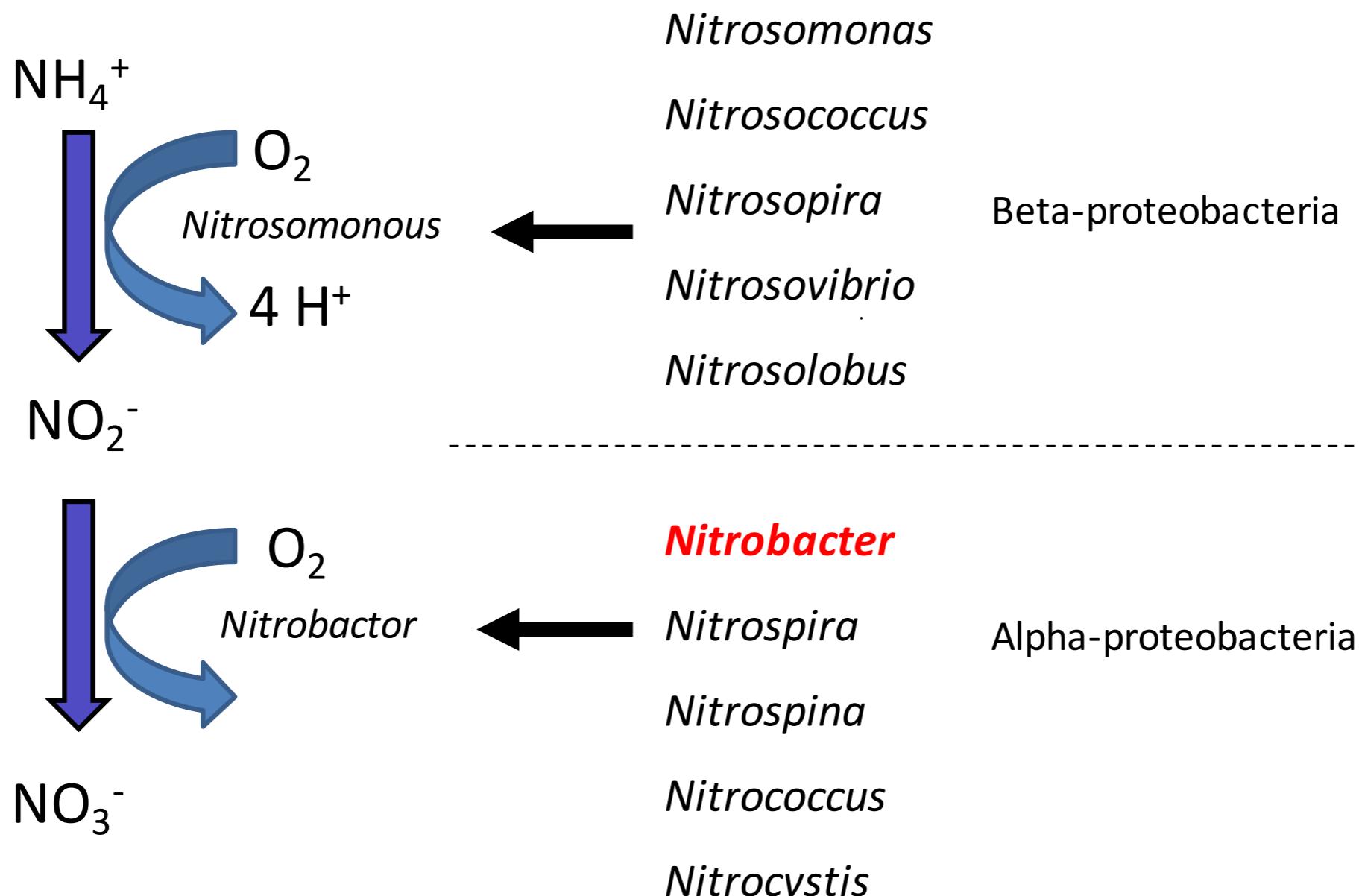
Comparison of methods: Wastewater treatment plant

Methods	Pros	Cons	Good / Bad for
Culture based (plates)	Easy, selective, quantitative	Slow, culture bias	Monitoring reactor, not clinical
Community characterization w/o identification (FAME)	Independent measure	Often inconclusive (not specific)	Tracking bioaugmentation, not new environment
Oligonucleotide probing (FISH)	spatial	Requires sequencing, sensitivity	Biofilm, structure, not monitoring at low concentration
Sequencing based methods coupled with PCR	comprehensive	Time, PCR bias	Characterize new environment, not biofilm structure or monitoring reactor
SSU, "Fingerprints"(T-RFLP, DGGE)	Faster, cheaper than Sequencing method	Coarse scale, PCR bias	Community response, not clinical
Activity (SMA)	Direct relevant	Coarse scale	Lab reactor, not food safety

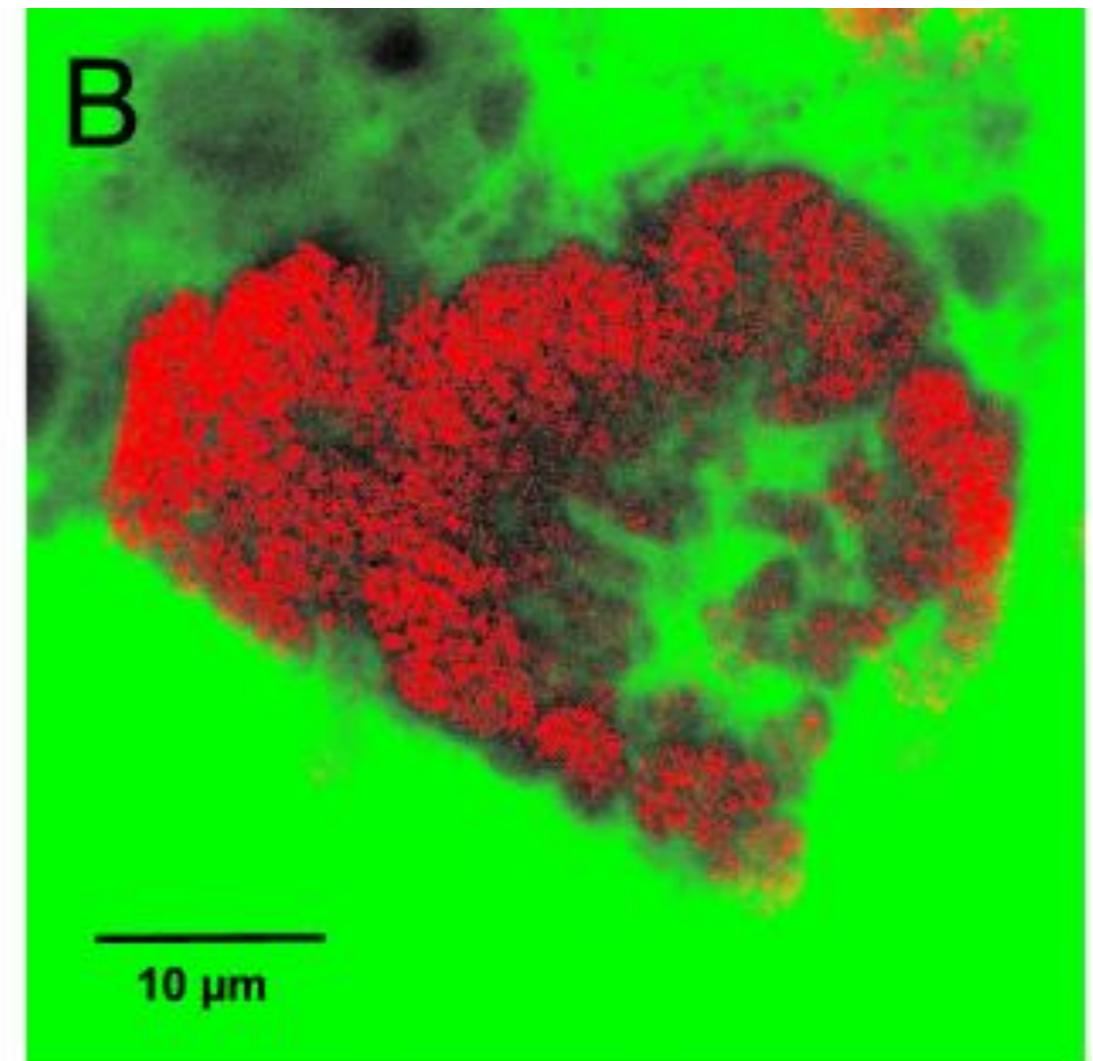
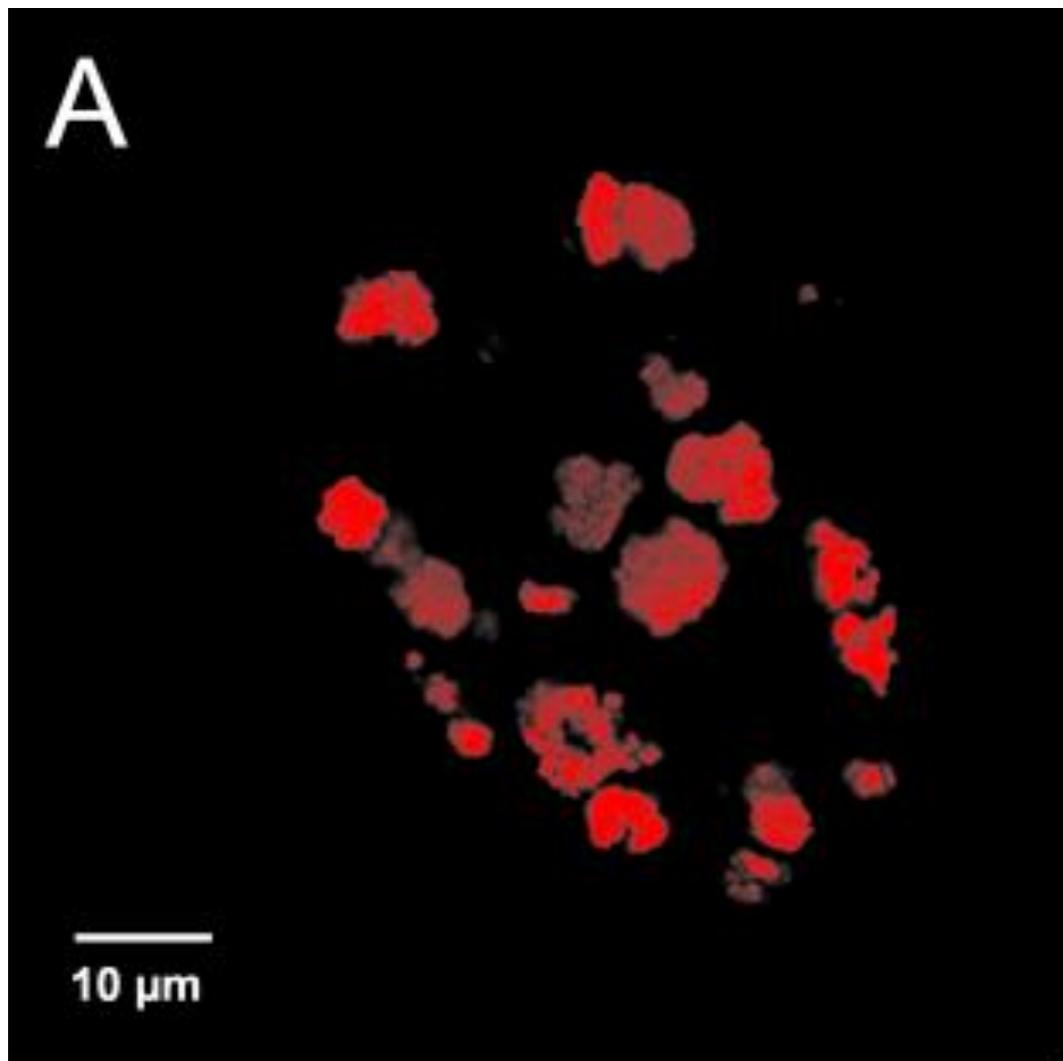
Case studies of molecular microbial techniques

Nitrification

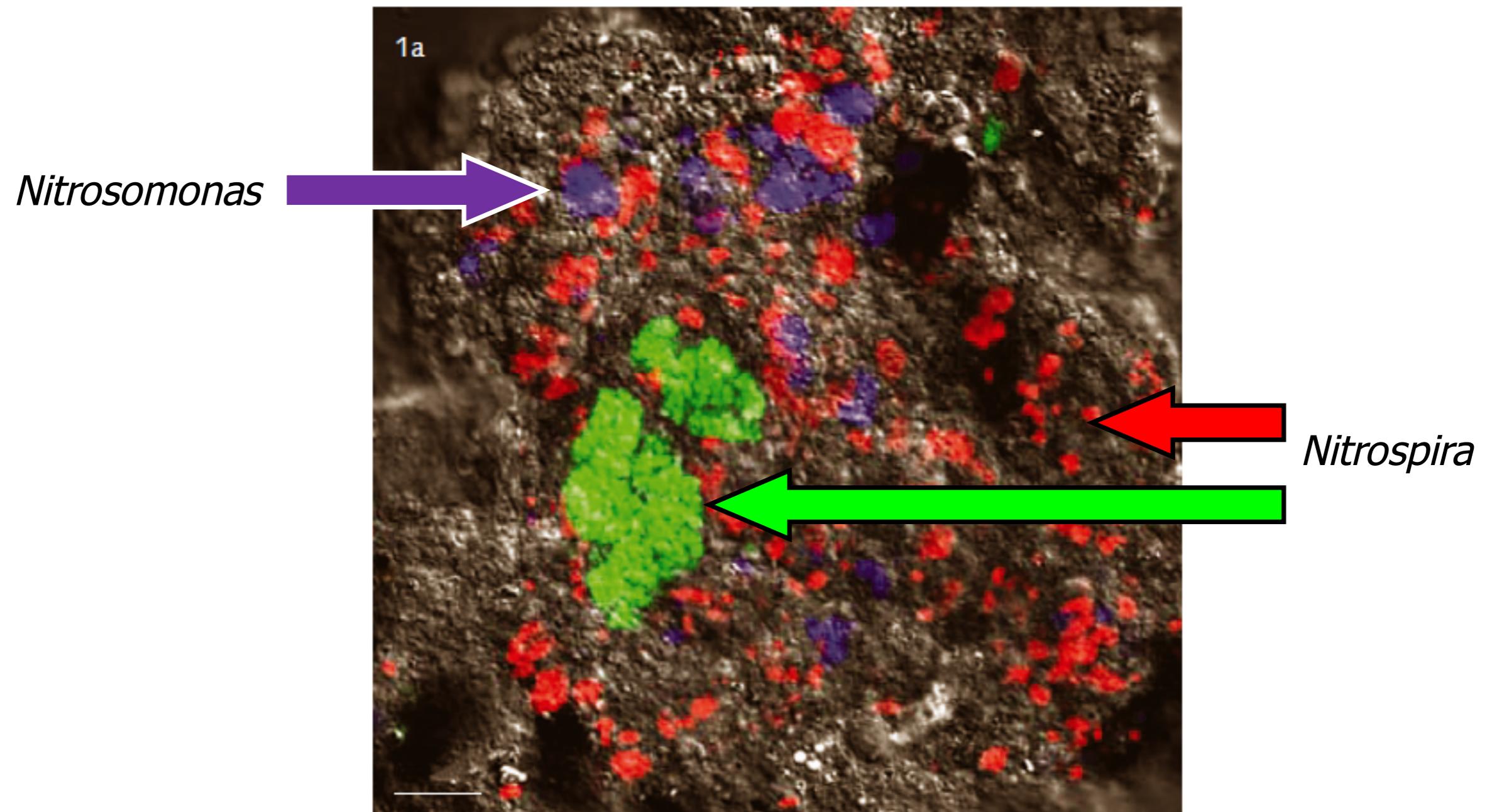
- *Nitrobacter spp.* were considered for to be responsible for nitrite oxidation in most activated sludge systems.



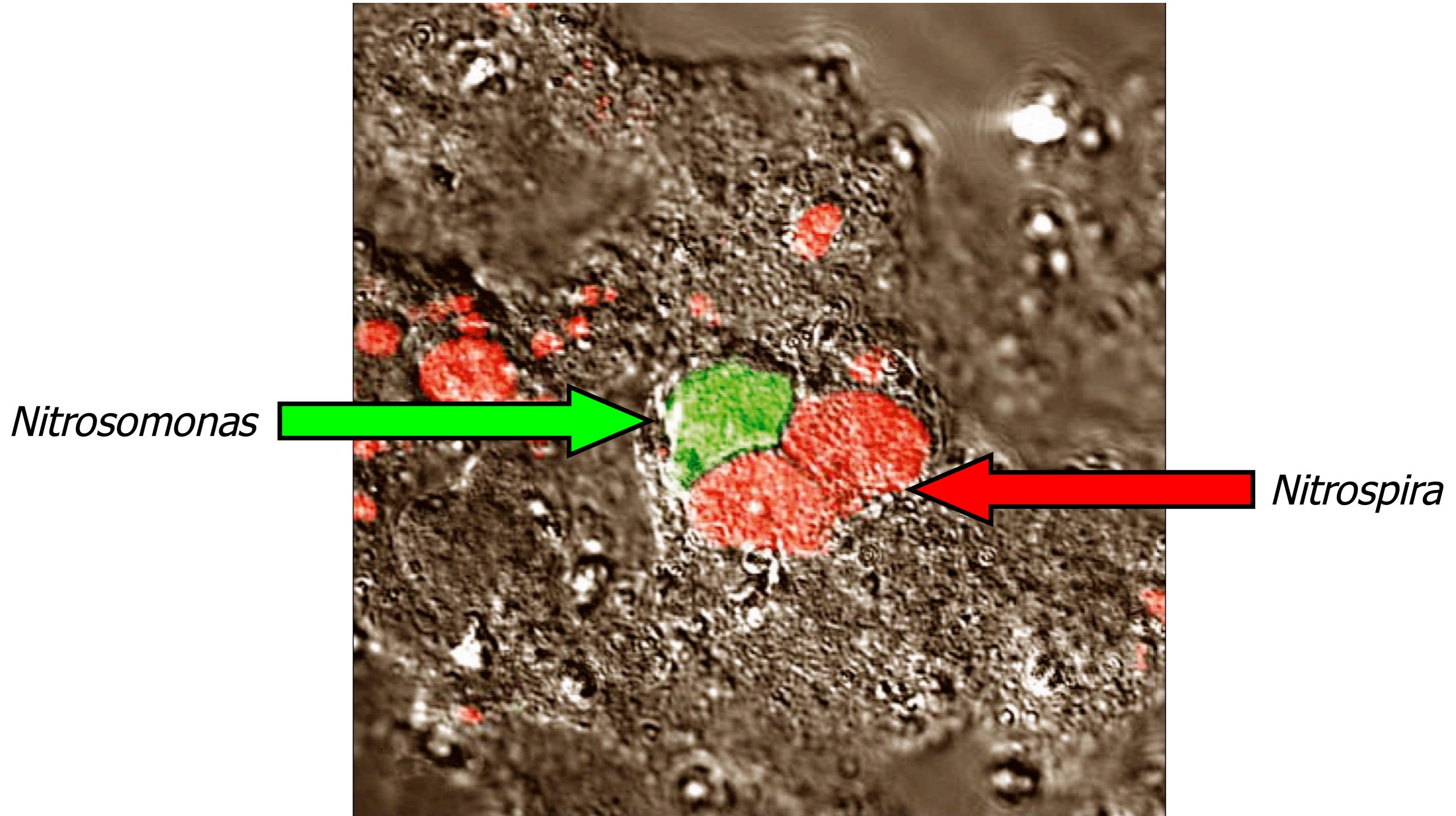
However, *Nitrospira*, but not nitrobacter was identified as the dominant nitrite oxidizer by FISH analysis!



FISH of nitrifying bacteria in activated sludge in one WWTP

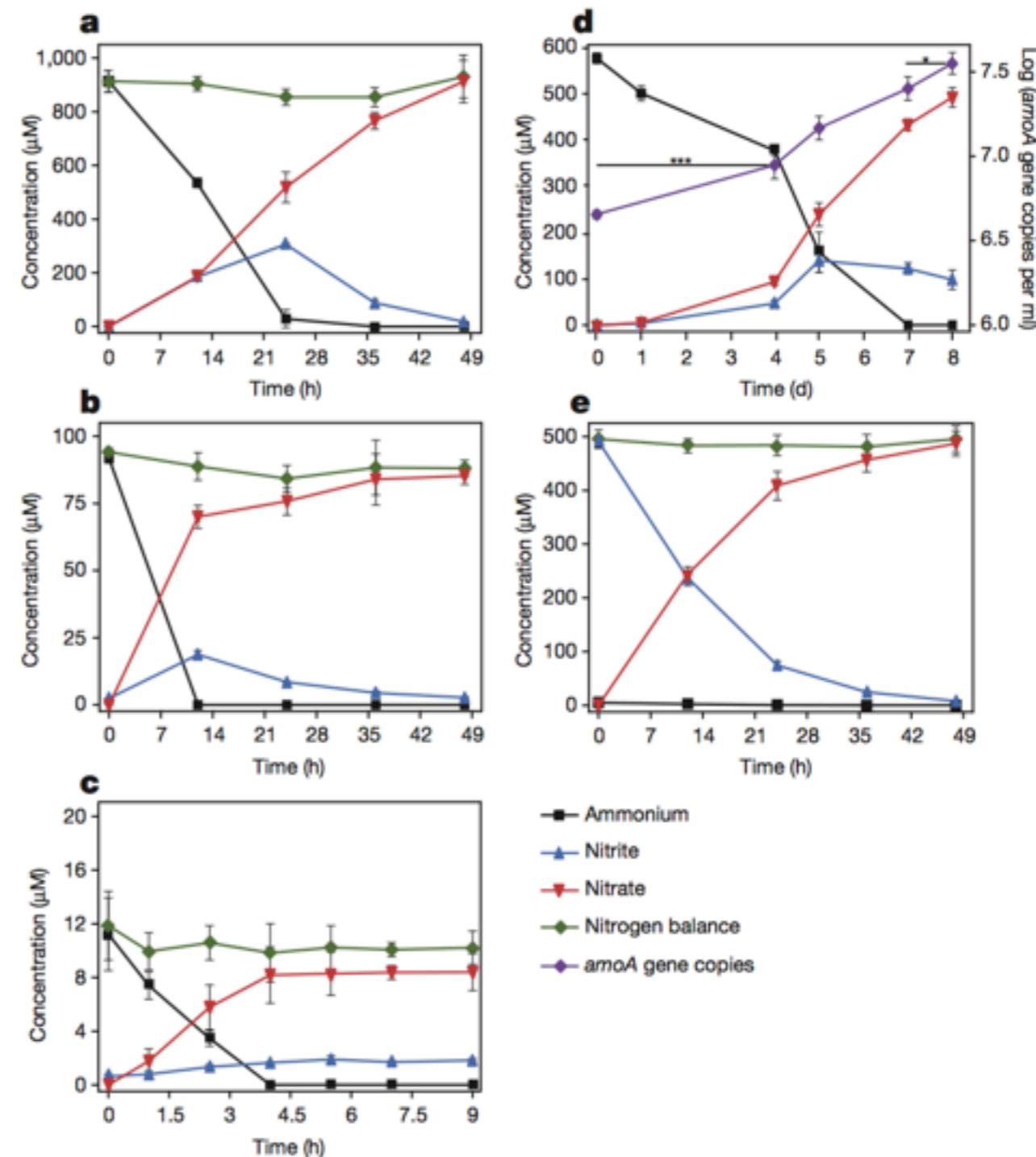


FISH of nitrifying bacteria in activated sludge in one WWTP



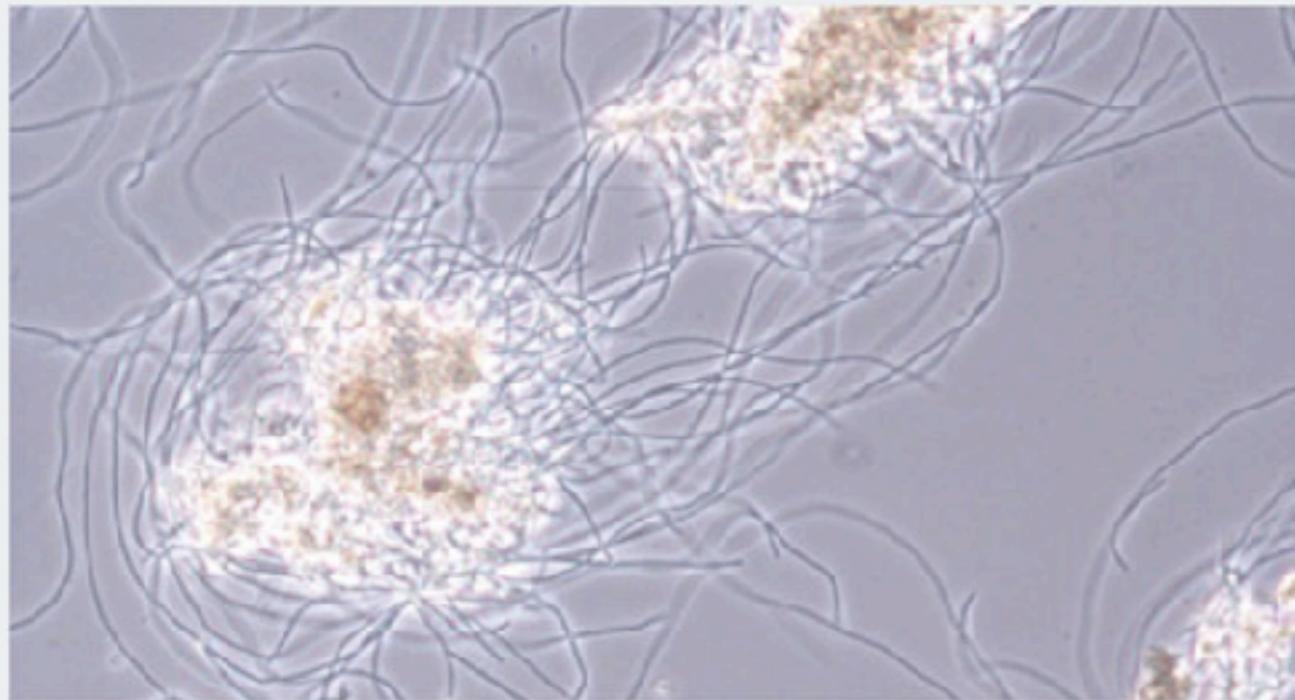
Complete Nitrification by single microorganism

- Comammox (COMplete AMMonia OXidiser) can first convert ammonia into nitrite and then into nitrate
- Complete nitrification would yield more energy ($\Delta G^\circ' = -349 \text{ kJ mol}^{-1} \text{ NH}_3$)
- Ammonia oxidation : $\Delta G^\circ' = -275 \text{ kJ mol}^{-1}$ for NH_3
- Nitrite oxidation : $\Delta G^\circ' = -74 \text{ kJ mol}^{-1} \text{ NO}_2^-$ for to nitrate



Decoding the DNA of wastewater

Identification by 16S rRNA Gene Amplicon Sequencing



Relative abundance [%]

Microthrix	11.8
C10_SBIA	9.1
Tetrasphaera	7.5
Dedchloromonas	3.1
B45	2.8
Trichococcus	2.5
Rhodoferax	1.4
P2CN44	1.3
Fodinbacter	1.1
Propionimonas	0.8
Hypomicrobium	0.8
Simplicispira	0.6
Gordonia	0.3
Sphingopyxis	0.3
Ferruginibacter	0.1
+1000 more...	



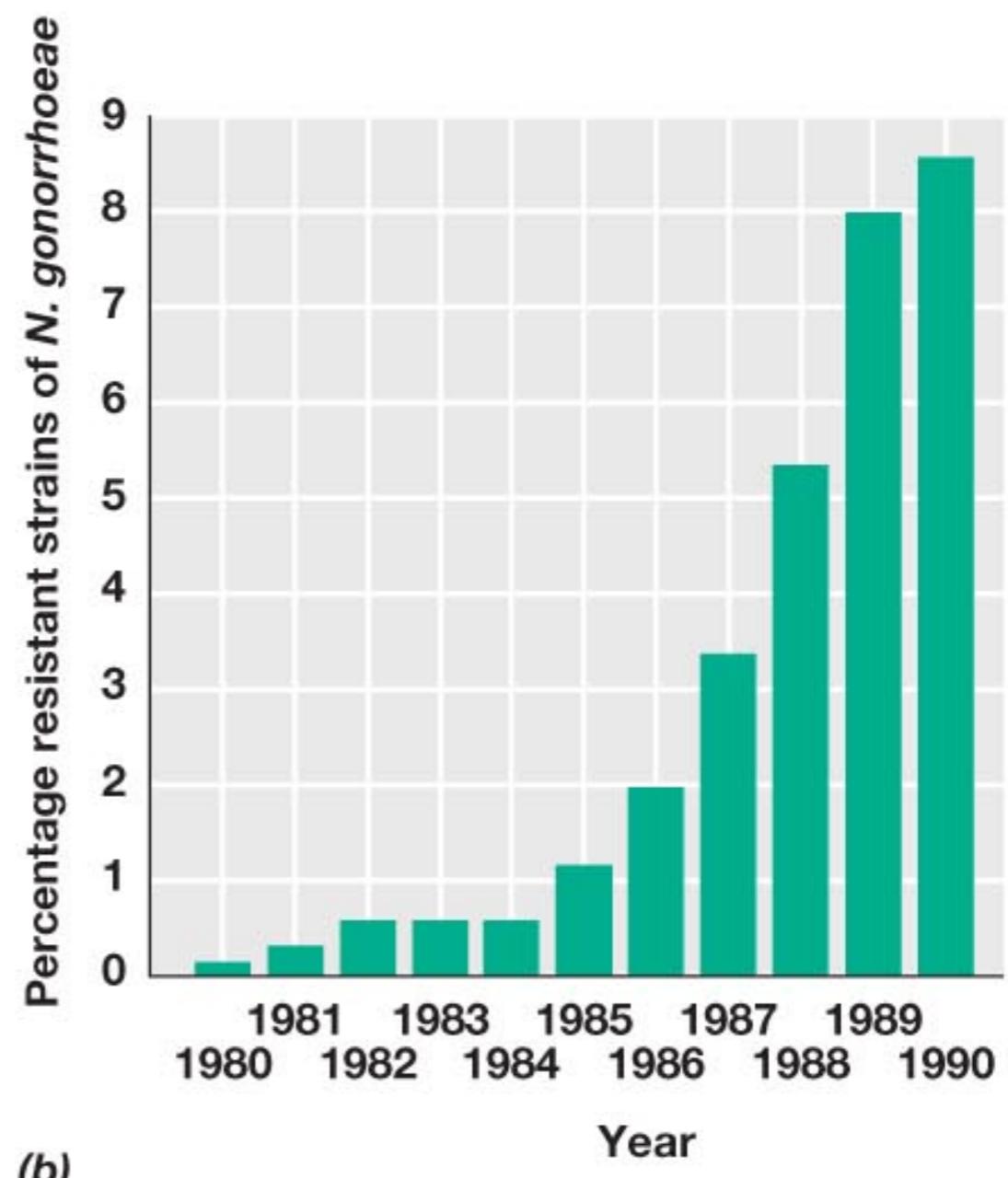
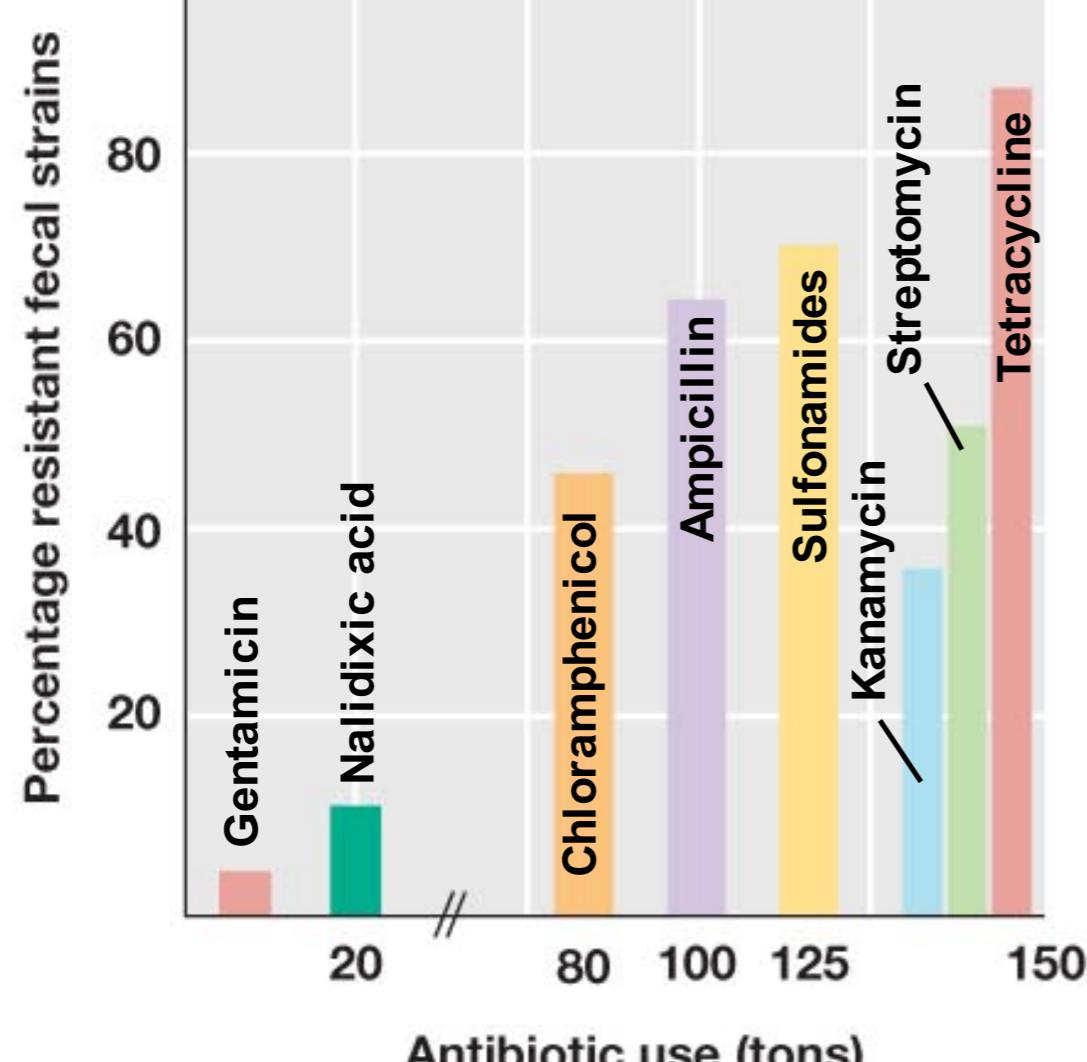
What are antimicrobials?

- Chemical substance that kill or inhibit microorganisms and cure infections

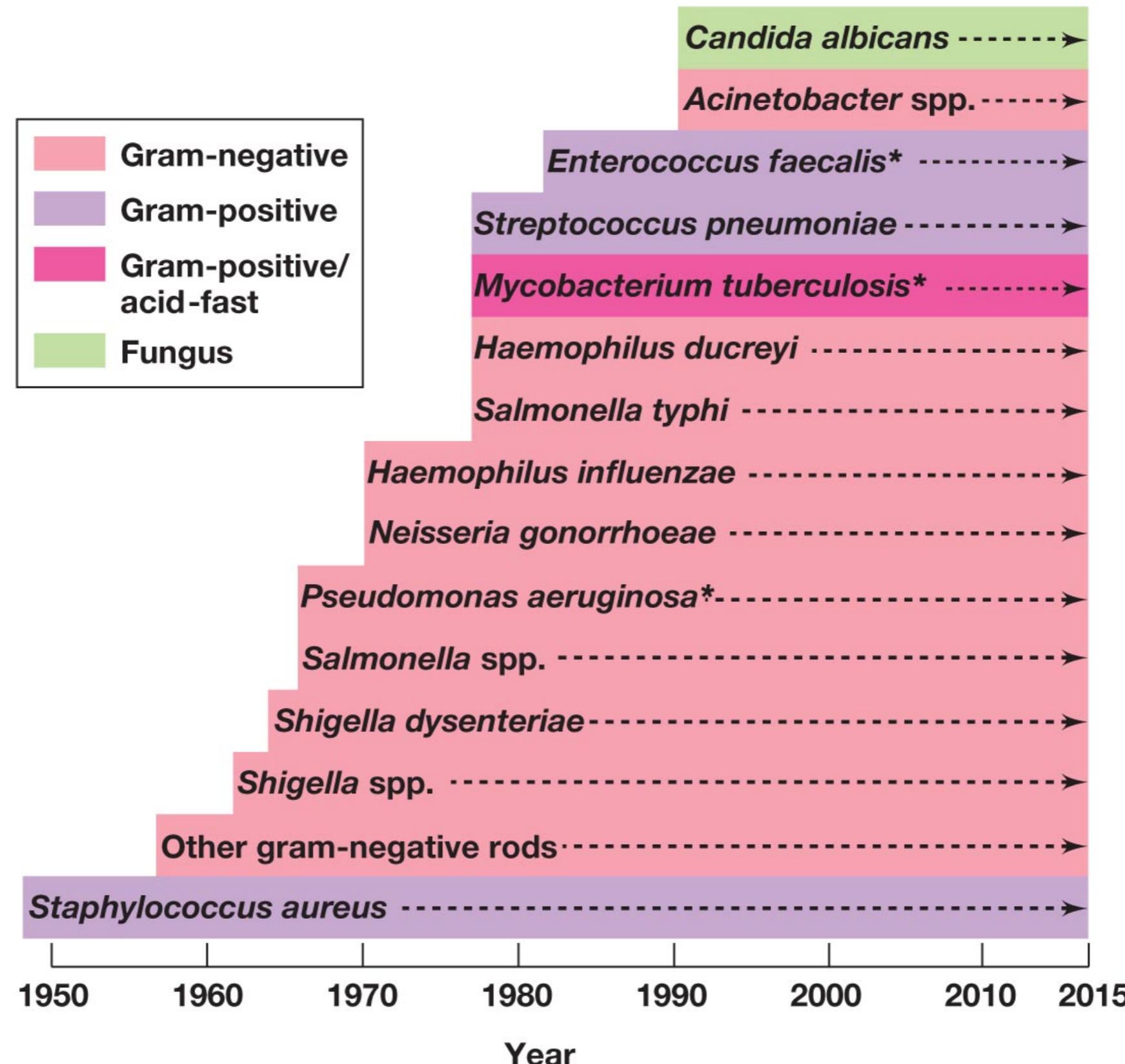


Source: <http://static.guim.co.uk/sys-images/Guardian/Pix/pictures/2011/11/18/1321605112780/Antibiotics-007.jpg>

Patterns of drug resistance in pathogens



The appearance of antimicrobial drug resistance in some human pathogens



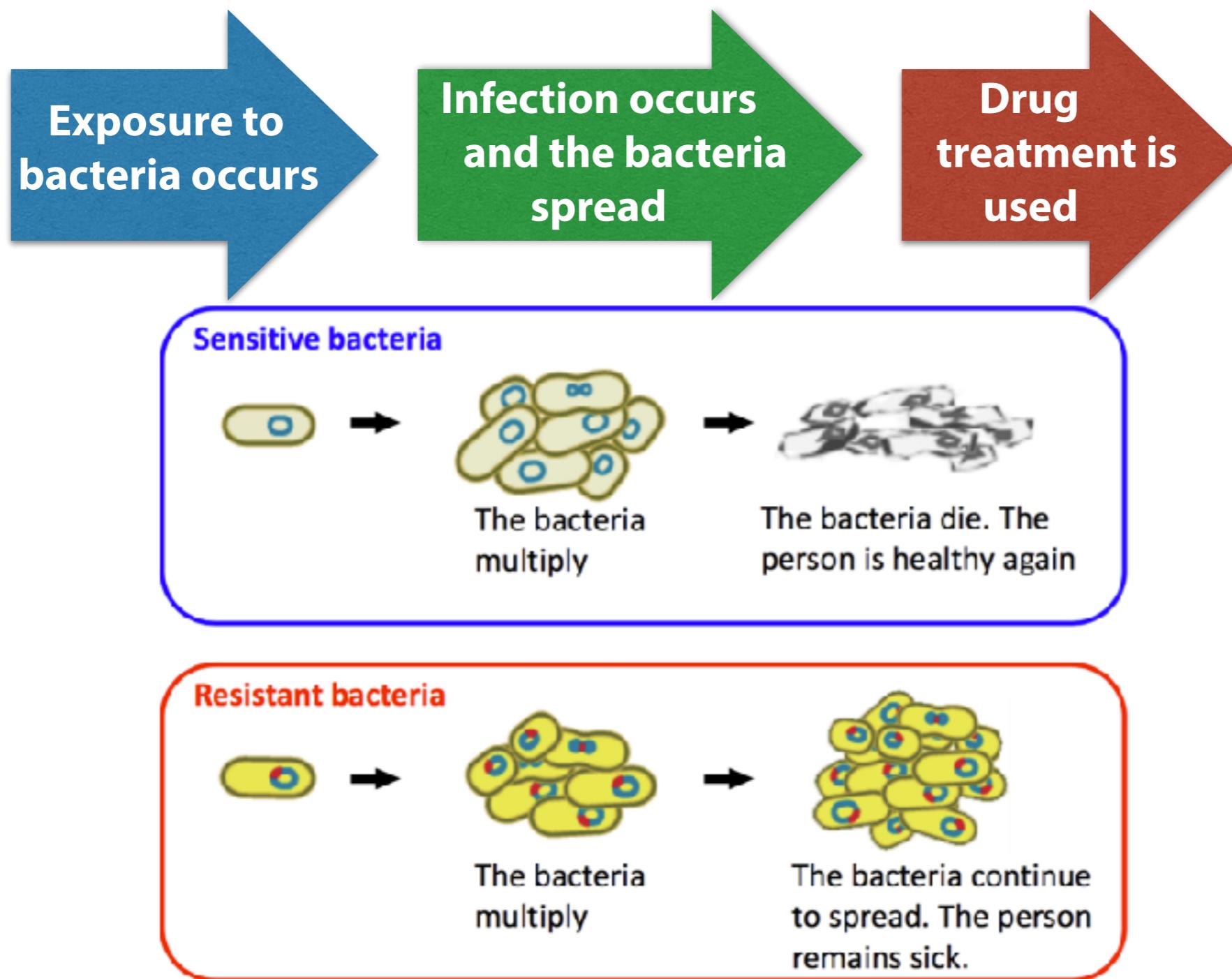
Organic wastewater contaminants - including antimicrobials - were in 80% of sampled US streams



Source: US Geological Survey, Koplin et al., Environ. Sci. Technol., 2002, 36, 1202 - 1211

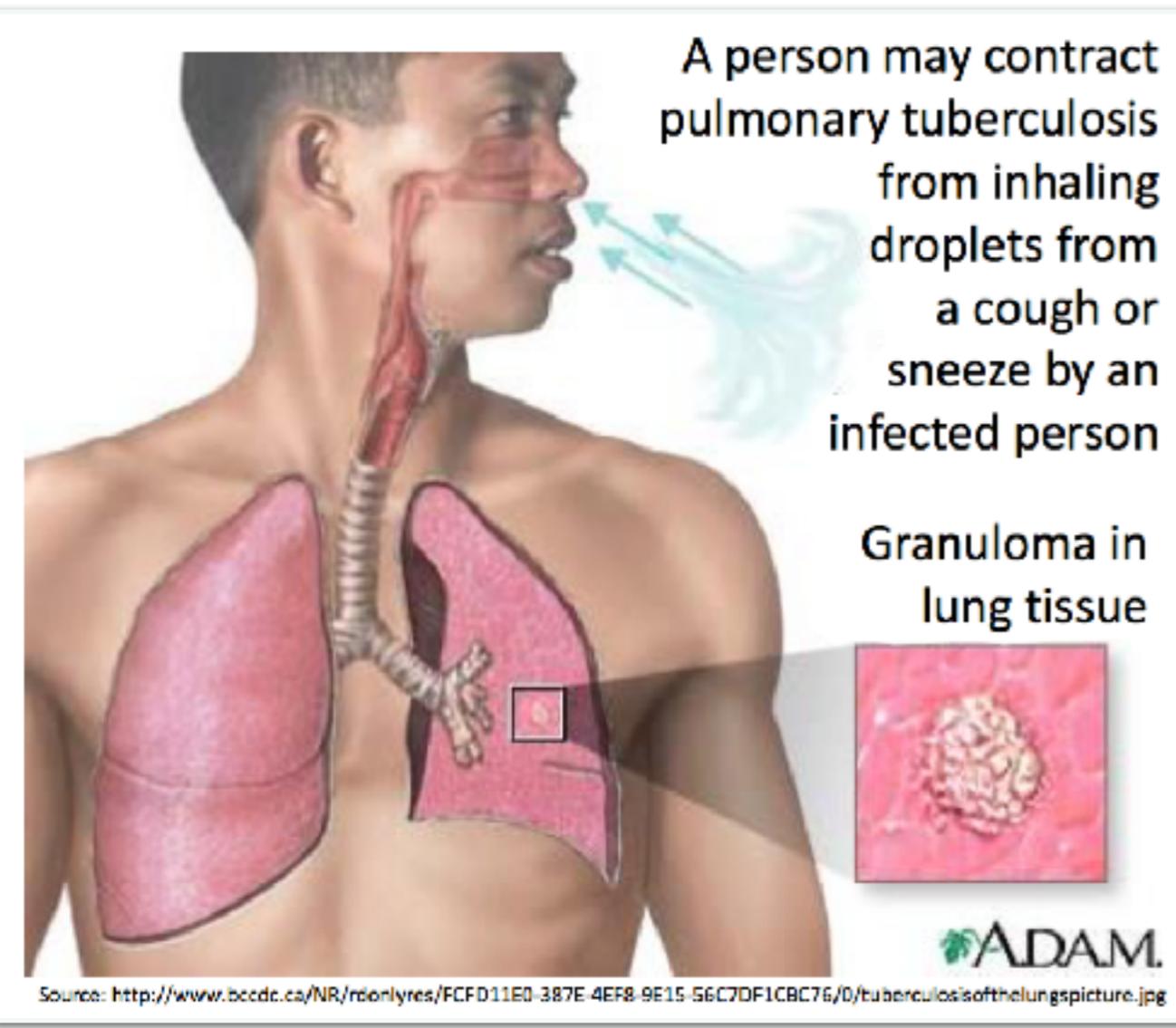
What is antimicrobial resistance?

- The ability of a microorganism to withstand antimicrobials



Source: NIAID <http://www3.niaid.nih.gov/topics/antimicrobialResistance/Understanding/drugResistanceDefinition.htm>

Antimicrobial resistance leads to therapeutic failures



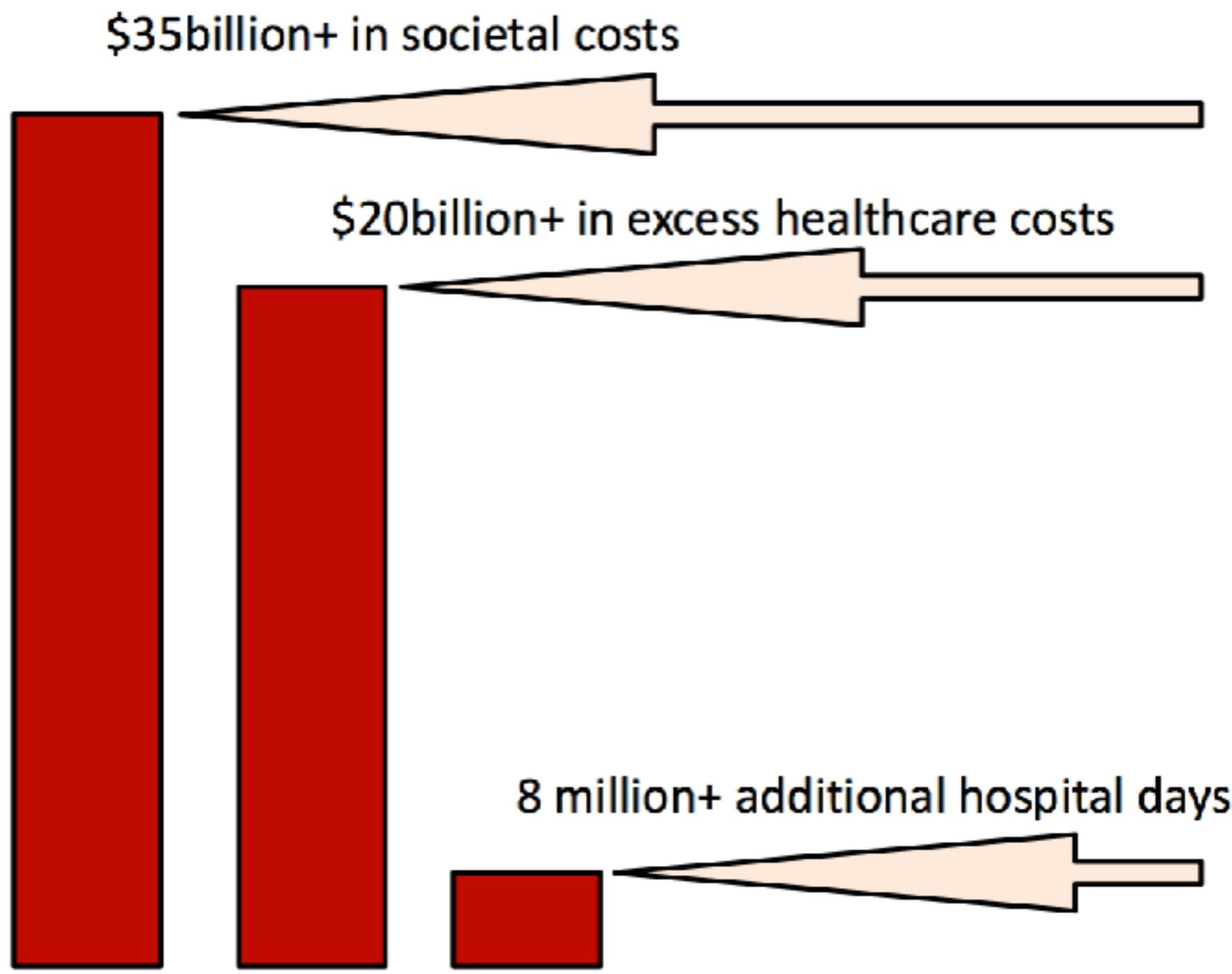
Tuberculosis
infects 7.5 million and kills 2.5 million
people annually, 10% patients carry highly
resistant bacteria (WHO)



Source: <http://www.bbc.co.uk/news/health-10930031>

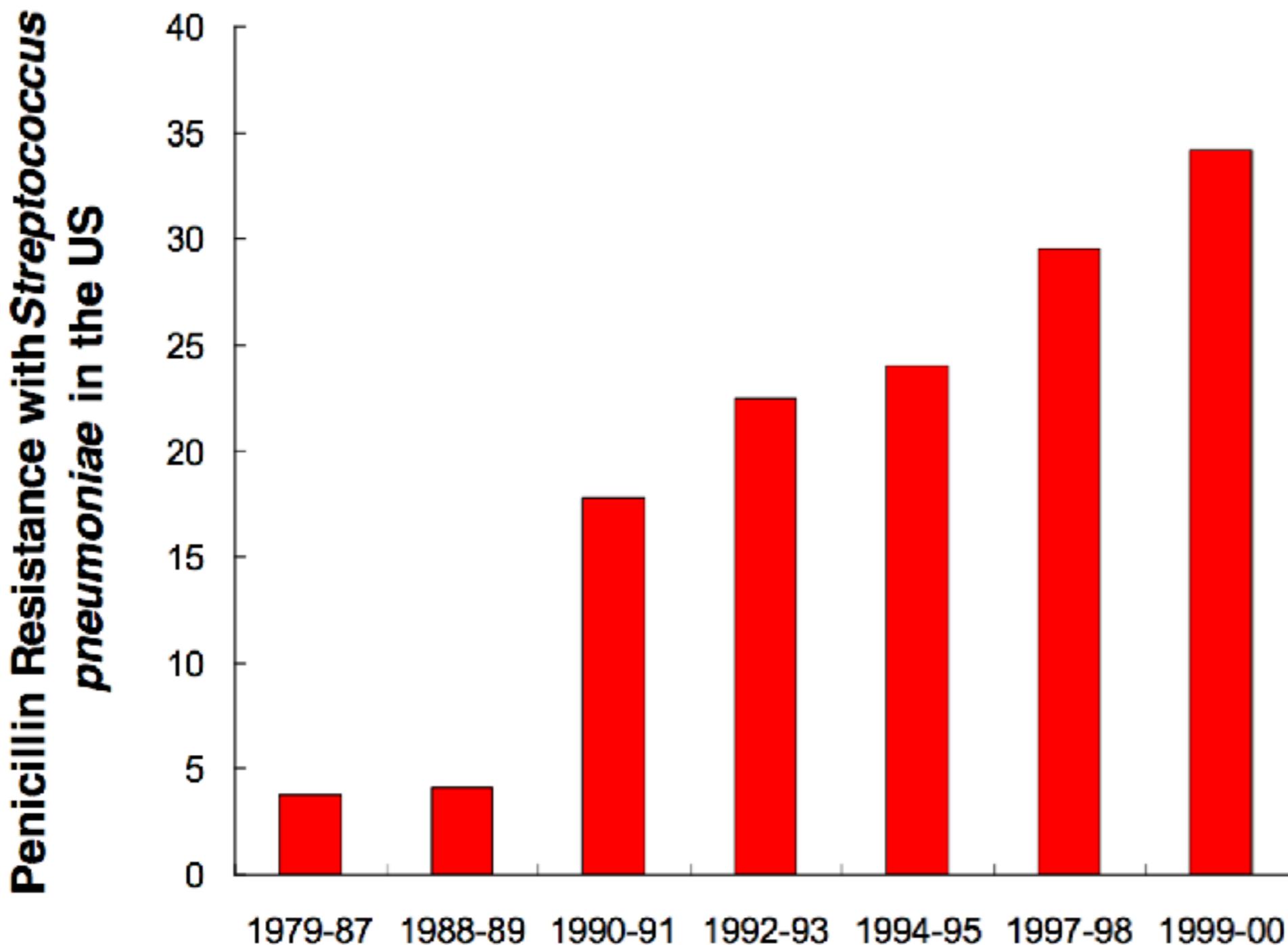
NDM-1 superbugs
“the doomsday scenario of a world
without antibiotics” (Prof. John
Conly)

Antimicrobial resistance leads to increased health care costs and hospital stays



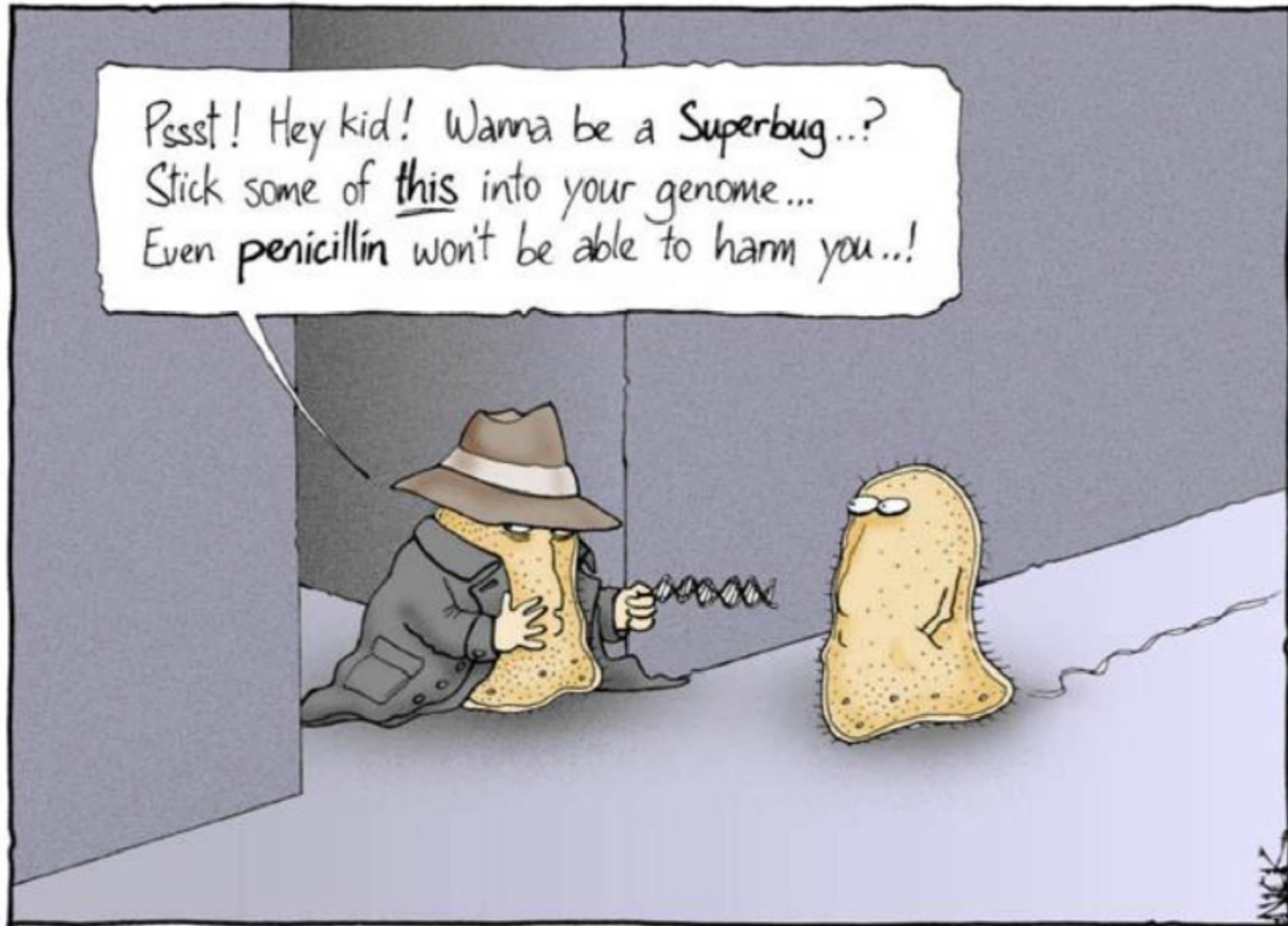
Source: http://www.cdc.gov/getsmart/healthcare/resources/factsheets/images/money_cost_graph_large.jpg

Antimicrobial resistance in pathogens increase rapidly



Source: Currents: Physician-To-Physician Clinical Resource, Spring 2004, Volume 5, Number 2

That's why antimicrobial resistance is popular among bacteria, but not us...



Pssst! Hey kid! Wanna be a Superbug...?
Stick some of this into your genome...
Even penicillin won't be able to harm you...!

It was on a short-cut through the hospital kitchens that Albert was first approached by a member of the Antibiotic Resistance

Large amount of antimicrobials are used in food animals for various purposes

- Large amount:
 - ~ 18 - 50 million lbs per year (UCS, AHI)
 - increased more than 100-fold from 1950 to 1999
- Purposes:
 - disease treatment
 - disease prevention
 - growth promotion



Exposure pathways from animals to humans

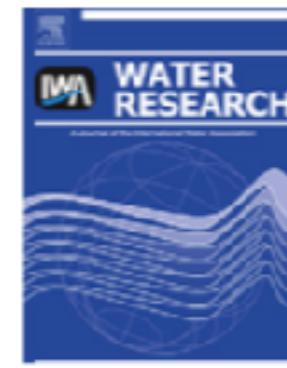
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Review

Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review

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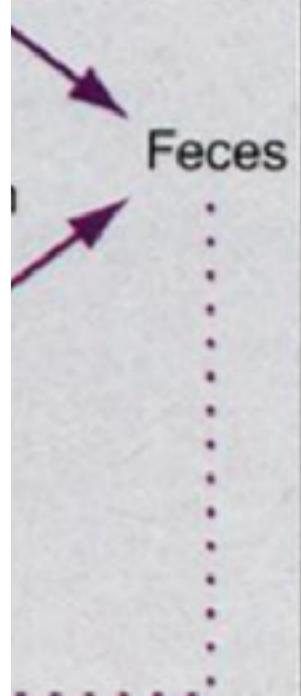
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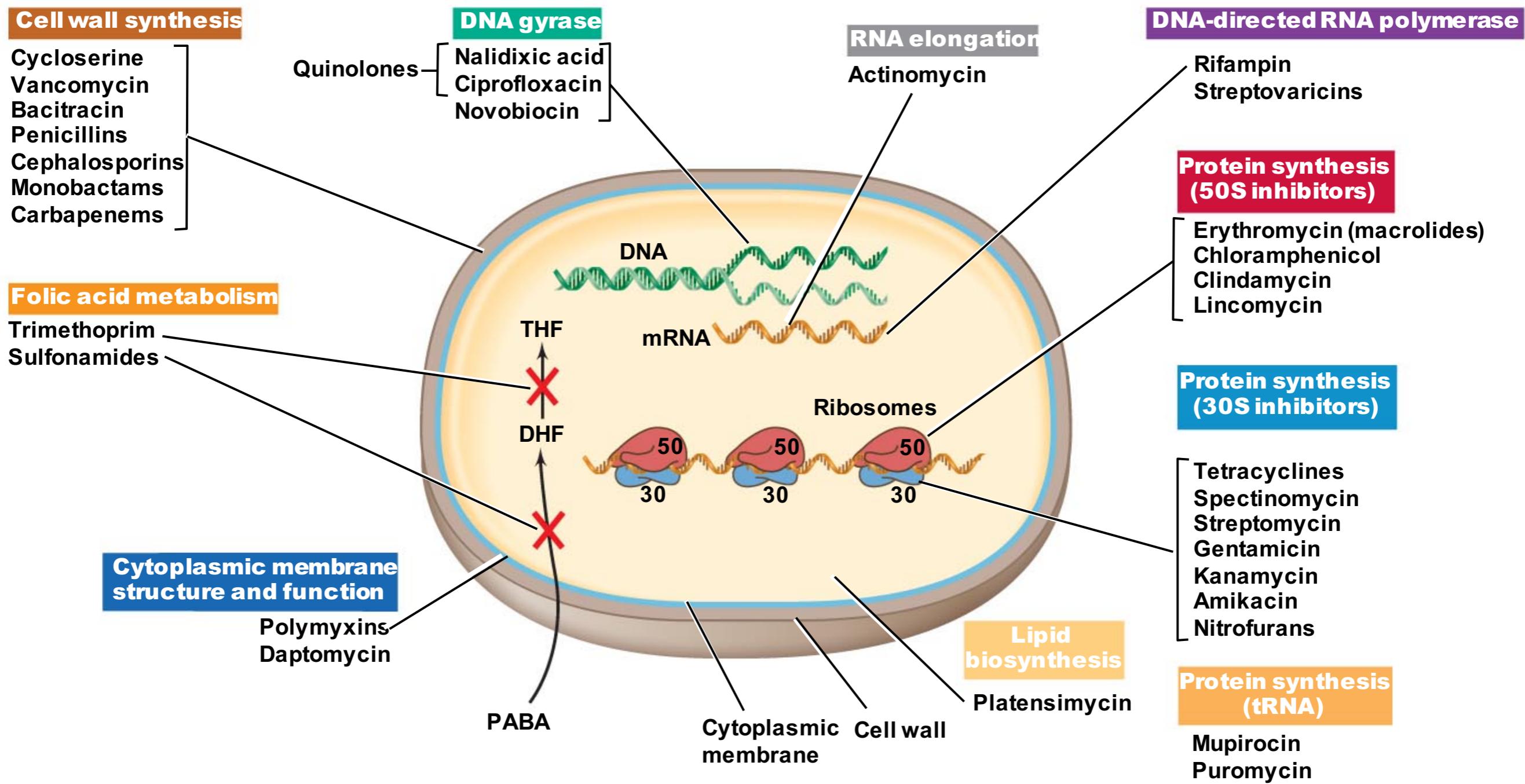
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Wolfgang et al., Science, 1998, V.279, p.996

Summary of antibiotics targets



Resistance Mechanisms and Spread

- At least five reasons that microorganisms are naturally resistant to certain antibiotics
- Organism is impermeable to antibiotic
- Organism can inactivate the antibiotic
- Organism may modify the target of the antibiotic
- Organism may develop a resistant biochemical pathway
- Organism may be able to pump out the antibiotic (efflux)

How bacteria acquire resistance? (genetic approach)

- Mutation
- Horizontal Gene Transfer
 - Transformation
 - Transduction
 - Conjugation

Mutation

- Mutation: Heritable change in DNA sequence that can lead to a change in phenotype (observable properties of an organism)
- Mutant : A strain of any cell or virus differing from parental strain in genotype (**nucleotide sequence of genome**)
- Wild-type strain: Typically refers to strain isolated from nature
- Genotype
 - Genetic information contained in the DNA of the organism
- Phenotype
 - Specific characteristics displayed by the organism
 - Auxotrophs: nutritionally deficient mutants
 - Prototrophs (wild type): normal, non-mutant forms

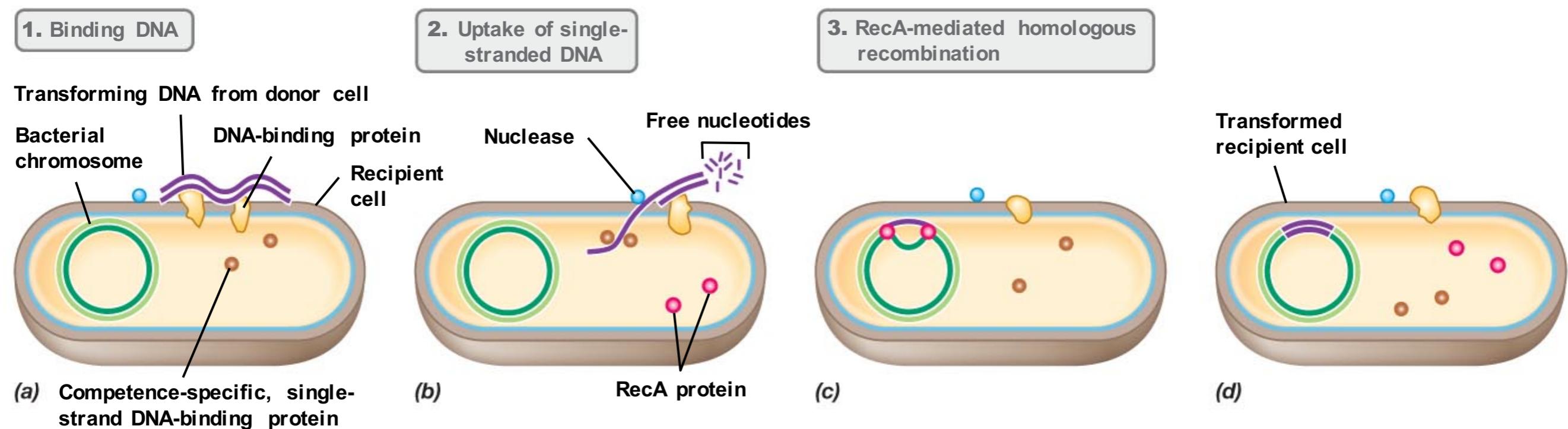
Horizontal Gene Transfer

- Transformation: Genetic transfer process by which DNA is incorporated into a recipient cell and brings about genetic change
- Transduction: Transformation of bacteria with DNA extracted from a bacterial virus
- Conjugation: mechanism of genetic transfer that involves cell-to-cell contact

Transformation

- Competent: cells are capable of taking up DNA and being transformed
 - In naturally transformable bacteria, competence is regulated
 - In other strains, specific procedures are necessary to make cells competent
 - Electricity can be used to force cells to take up DNA (electroporation)
- During natural transformation, integration of transforming DNA is a highly regulated, multistep process

Mechanism of transformation in a gram-positive bacterium

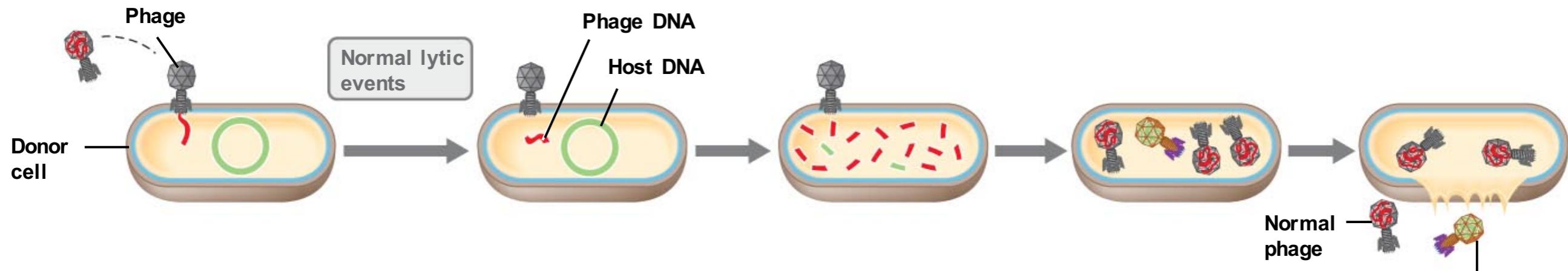


Transfection

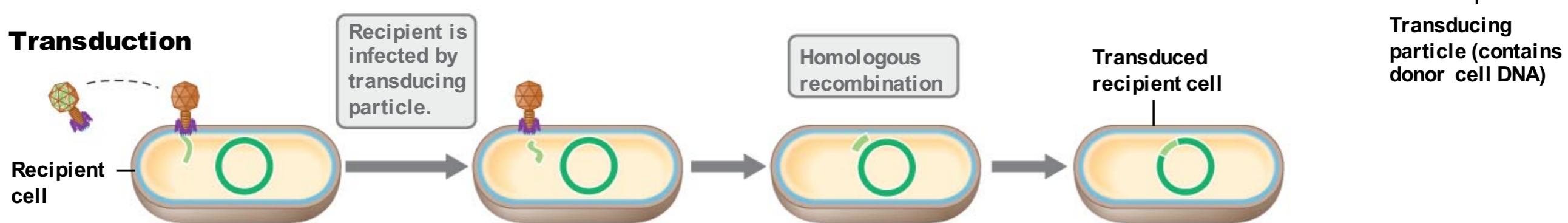
- Transfer of DNA from one cell to another by a bacteriophage
- Two modes
 - Generalized transduction: DNA from any portion of the host genome is packaged inside the virion
 - Specialized transduction: DNA from a specific region of the host chromosome is integrated directly into the virus genome

Generalized transduction

Lytic cycle



Transduction

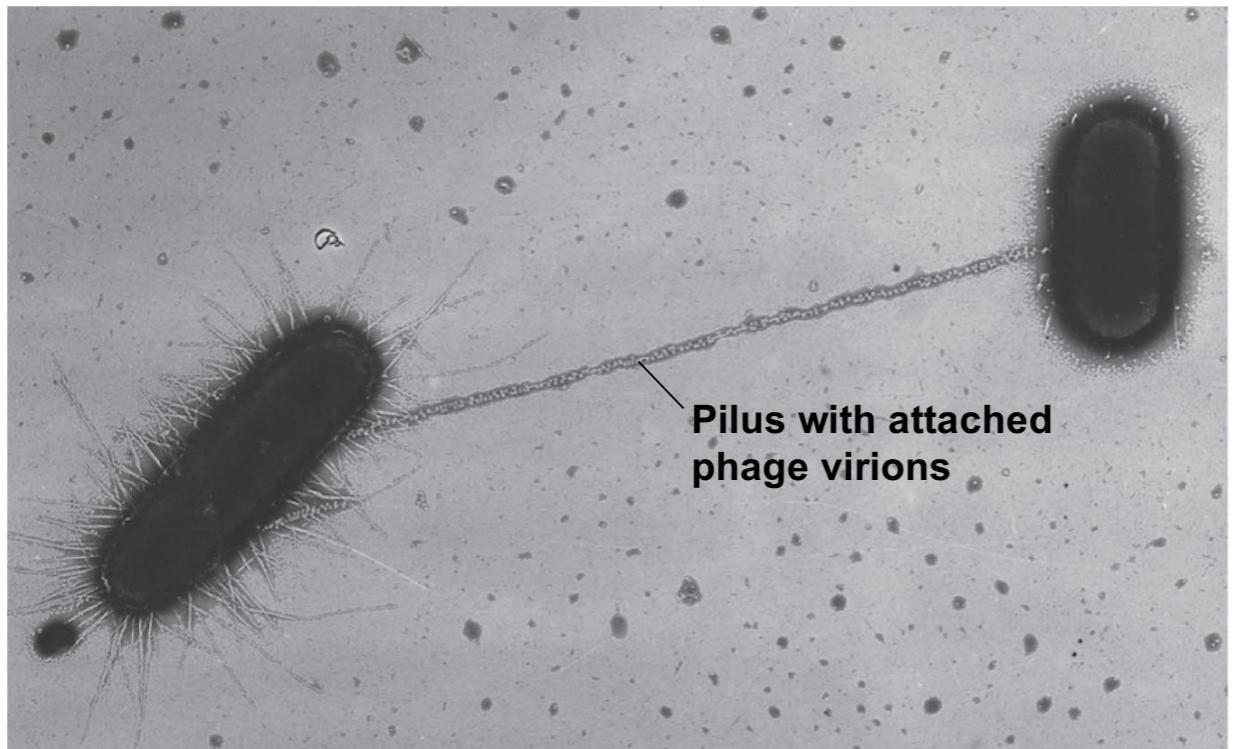


- DNA derived from virtually any portion of the host genome is packaged inside the mature virion
 - Defective virus particle incorporates fragment of the cell's chromosome randomly
 - Virus can be temperate or virulent
 - Low efficiency

Transfer of plasmid DNA by conjugation

Plasmid-encoded mechanism

- Donor cell: contains conjugative plasmid
- Recipient cell: does not contain plasmid



C. Brinton

