Chapter 22
Methods in Microbial Ecology

I. Culture-Dependent Analyses of Microbial Communities

- 22.1 Enrichment
- 22.2 Isolation
22.1 Enrichment

- **Isolation**
  - The separation of individual organisms from the mixed community

- **Enrichment Cultures**
  - Select for desired organisms through manipulation of medium and incubation conditions

- **Inocula (singular Inoculum)**
  - The sample from which microorganisms will be isolated

**Figure 22.1** The isolation of *Azotobacter*.

Selection for aerobic $\text{N}_2$-fixing bacteria usually results in the isolation of *Azotobacter* or its relatives. By contrast, enrichment with fixed forms of nitrogen such as $\text{NH}_4^+$ rarely results in isolating nitrogen-fixing bacteria because there is no selective pressure for nitrogen fixation.
22.1 Enrichment

- Enrichment Cultures
  - Can prove the presence of an organism in a habitat
  - Cannot prove an organism does not inhabit an environment
- The ability to isolate an organism from an environment says nothing about its ecological significance

22.1 Enrichment

- **The Winogradsky Column**
  - An artificial microbial ecosystem (Figure 22.2)
  - Serves as a long-term source of bacteria for enrichment cultures
  - Named for Sergei Winogradsky
  - First used in late 19th century to study soil microorganisms
22.1 Enrichment

**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*
22.2 Isolation

- **Pure cultures** contain a single kind of microorganism
  - Can be obtained by streak plate, agar shake, or liquid dilution (Figure 22.3)
- **Agar dilution tubes** are mixed cultures diluted in molten agar
  - Useful for purifying anaerobic organisms

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**Figure 22.3 Pure culture methods**

- Organisms that form distinct colonies on plates are usually easy to purify.
- Colonies of phototrophic purple bacteria in agar dilution tubes: the molten agar was cooled to app. 45°C before inoculation.
- A dilution series was established from left to right, eventually yielding well-isolated colonies.
- The tubes were sealed with a 1:1 mixture of sterile paraffin and mineral oil to maintain anaerobiosis
22.2 Isolation

- **Most-probable-number technique**
  - Serial 10× dilutions of inocula in a liquid media
  - Used to estimate number of microorganisms in food, wastewater and other samples (Figure 22.4)
22.2 Isolation

- **Axenic culture** (grown under sterile conditions) can be verified by
  - Microscopy
  - Observation of colony characteristics
  - Tests of the culture for growth in other media

- **Laser tweezers** are useful for
  - Isolating slow-growing bacteria from mixed cultures (Figure 22.5)

![Figure 22.5 The laser tweezers for the isolation of single cells](image)

- A laser beam strongly focused on a very small object such as a cell creates downward radiation forces that allow the cell to be dragged in any direction.
- The laser beam can lock onto a single cell present in a mixture in a capillary tube and drag the optically trapped cell away from the other cells.
- Once the desired cell is far enough away from the other cells, the capillary is severed and the cell is flushed into a tube of sterile medium.
22.2 Isolation

- **Flow cytometry**
  - Uses lasers
  - Suspended cultures passed through specialized detector
  - Cells separated based on fluorescence

II. Culture-Independent Analyses of Microbial Communities

- 22.3 General Staining Methods
- 22.4 Fluorescent *In Situ* Hybridization (FISH)
- 22.5 PCR Methods of Microbial Community Analysis
- 22.6 Microarrays and Microbial Diversity: Phylochips
- 22.7 Environmental Genomics and Related Methods
22.3 General Staining Methods

- Fluorescent staining using **DAPI** or **acridine orange (AO)**
  - DAPI-stained cells fluoresce bright blue (Figure 22.6a)
  - AO-stained cells fluoresce orange or greenish-orange (Figure 22.6b)
  - DAPI and AO fluoresce under UV light
  - DAPI and AO are used for the enumeration of microorganisms in samples
  - DAPI and AO are nonspecific and stain nucleic acids
  - Cannot differentiate between live and dead cells

Figure 22.6 Nonspecific fluorescent stains

(a) DAPI and (b) acridine orange (AO) staining showing microbial communities inhabiting activated sludge in a municipal wastewater treatment plant. With acridine orange, cells containing low RNA levels stain green.

(c) SYBR Green–stained sample of Puget Sound (Washington, USA) surface water showing green-fluorescing bacterial cells. The large cells near the center of the field are 0.8–1.0 m in diameter
22.3 General Staining Methods

- **Viability stains**: differentiate between live and dead cells (Figure 22.7)
  - 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

*Figure 22.7 Viability staining*

Live (green) and dead (red) cells of *Micrococcus luteus* (cocci) and *Bacillus cereus* (rods) stained by the LIVE/DEAD BacLight Bacterial Viability Stain
22.3 General Staining Methods

- **Fluorescent antibodies** can be used as a cell tag
  - Highly specific
  - Making antibodies is time consuming and expensive
- **Green fluorescent protein** can be genetically engineered into cells to make them **autofluorescent**
  - Can be used to track bacteria
  - Can act as a reporter gene

22.4 Fluorescent *In Situ* Hybridization (FISH)

- **Nucleic acid probe** is DNA or RNA complementary to a sequence in a target gene or RNA
- **FISH**: fluorescent *in situ* hybridization (Figure 22.9)
  - Phylogenetics of microbial populations (Figure 22.10)
  - Used in microbial ecology, food industry, and clinical diagnostics
  - **CARD-FISH**
The photomicrographs shown here, produced by (a) phase-contrast and (b) a technique called phylogenetic FISH, are of the same field of cells. Although the large oval cells are of a rather unusual morphology and size for prokaryotic cells and all look similar in phase-contrast microscopy, the phylogenetic stains reveal that there are two genetically distinct types (one stains yellow and one stains blue).

(a) Nitrifying bacteria. Red, ammonia-oxidizing bacteria; green, nitrite-oxidizing bacteria.
(b) Confocal laser scanning micrograph of a sewage sludge sample. The sample was treated with three phylogenetic FISH probes, each containing a fluorescent dye (green, red, or purple) that identifies a particular group of Proteobacteria. Green-, red- or purple-stained cells reacted with only a single probe; other cells reacted with multiple probes to give blue or yellow.
22.4 Fluorescent *In Situ* Hybridization (FISH)

- **CARD-FISH**
  - FISH can be used to measure gene expression in organisms in a natural sample (Figure 22.11)
  - A FISH method that enhances the signal is called *catalyzed reporter deposition FISH (CARD-FISH)*

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**Figure 22.11** Catalyzed reporter deposition FISH (CARD-FISH) labeling of Archaea

Archaeal cells in this preparation fluoresce intensely (green) relative to DAPI-stained cells (blue)
22.5 PCR Methods of Microbial Community Analysis

- Specific genes can be used as a measure of diversity
  - Techniques used in molecular biodiversity studies (Figure 22.12)
    - DNA isolation and sequencing
    - PCR
    - Restriction enzyme digest
    - Electrophoresis
    - Molecular cloning

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**Figure 22.12** Steps in single-gene biodiversity analysis of a microbial community

1. Extract total community DNA
2. Amplify by PCR using fluorescently tagged primers
3. Restriction enzyme digest and run on gel
4. Amplify 16S RNA genes using general primers (for example, Bacteria-specific) or more restrictive primers (to target endospore-forming bacteria)
5. Excise bands and clone 16S rRNA genes
6. Sequence
7. Generate tree from results using endospore-specific primers

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22.5 PCR Methods of Microbial Community Analysis

- **DGGE**: denaturing gradient gel electrophoresis separates genes of the same size based on differences in base sequence (Figure 22.13)
  - Denaturant is a mixture of urea and formamide
  - Strands melt at different denaturant concentrations

Figure 22.13 PCR and DGGE gels

(a) PCR amplification

(b) DGGE
22.5 PCR Methods of Microbial Community Analysis

- **T-RFLP**: terminal restriction fragment length polymorphism
  - Target gene is amplified by PCR
  - Restriction enzymes are used to cut the PCR products
- **ARISA**: automated ribosomal intergenic spacer analysis
  - Related to T-RFLP
  - Uses DNA sequencing

22.5 PCR Methods of Microbial Community Analysis

- 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 less than 0.1% of bacteria have been cultured
22.6 Microarrays and Microbial Diversity: Phylochips

- **Phylochip**: microarray that focuses on phylogenetic members of microbial community (Figure 22.15)
  - Circumvents time-consuming steps of DGGE and T-RFLP

![Figure 22.15 Phylochip analysis of sulfate-reducing bacteria diversity](image)

Each spot on the microarray shown has an oligonucleotide complementary to a sequence in the 16S rRNA of a different species of sulfate-reducing bacteria. After the microarray is hybridized with 16S rRNA genes PCR amplified from a microbial community and then fluorescently labeled, the presence or absence of each species is signaled by fluorescence (positive or weak positive) or nonfluorescence (negative), respectively.
22.7 Environmental Genomics and Related Methods

- *Environmental genomics (metagenomics)*
  - 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

III. Measuring Microbial Activities in Nature

- 22.8 Chemical Assays, Radioisotopic Methods, and Microelectrodes
- 22.9 Stable Isotopes
- 22.10 Linking Specific Genes and Functions to Specific Organisms
22.8 Chemical Assays, Radioisotopes, & Microelectrodes

- In many studies, direct chemical measurements are sufficient (Figure 22.18)
  - Higher sensitivity can be achieved with radioisotopes
  - Proper *killed cell* controls must be used

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**Figure 22.18** Microbial activity measurements

- **Chemical measurement:** Lactate and $\text{H}_2\text{S}$ transformations during sulfate reduction.
  - Sulfate reduction
  - Photosynthesis

- **Radioisotopic measurement:** sulfate reduction measured with $^{35}\text{SO}_4^{2-}$
  - Sulfate reduction
  - $^{14}\text{C}$-Glucose respiration

- **Radioisotopic measurement:** production of $^{14}\text{CO}_2$ from $^{14}\text{C}$-glucose.
  - $^{14}\text{CO}_2$ evolution
22.8 Chemical Assays, Radioisotopes, & Microelectrodes

- **Microelectrodes**
  - Can measure a wide range of activity
  - pH, oxygen, CO₂, and others can be measured
  - Small glass electrodes, quite fragile (Figure 22.19)
  - Electrodes are carefully inserted into the habitat (e.g., microbial mats)
    - Measurements taken every 50–100μm (Figure 22.20)

The platinum rod functions as a cathode and when voltage is applied, O₂ is reduced to H₂O, generating a current. The current resulting from the reduction of O₂ at the gold surface of the cathode is proportional to the O₂ concentration in the sample. Note the scale of the electrode.

![Figure 22.19 Microelectrodes](image)

(b) Biological microsensor for the detection of nitrate (NO₃⁻). Bacteria immobilized at the sensor tip denitrify NO₃⁻ (or NO₂⁻) to N₂O, which is detected by reduction to N₂ at the cathode.
22.9 Stable Isotopes

- Nonradioactive isotopes of an element
  - Used to study microbial transformations in nature
  - *Isotope fractionation*
    - Carbon and sulfur are commonly used
    - Lighter isotope is incorporated preferentially over heavy isotope (Figure 22.22)
    - Indicative of biotic processes
    - Isotopic composition reveals its past biology (e.g., carbon in plants and petroleum)
    - The activity of sulfate-reducing bacteria is easy to recognize from their fractionation of sulfur in sulfides
22.10 Linking Specific Genes and Functions to Specific Organisms

- Flow cytometry and multiparametric analysis
  - Natural communities contain large populations
  - Flow cytometer examines specific cell parameters very fast (Figure 22.26)
    - Cell size
    - Cell shape
    - Fluorescence
  - Parameters can be combined and analyzed (multiparametric analysis)
22.10 Linking Specific Genes and Functions to Specific Organisms

- Radioisotopes used as measures of microbial activity in a microscopic technique called *microautoradiography (MAR)*
- Radioisotopes can also be used with FISH
  - *FISH microautoradiography (FISH-MAR)*
    - Combines phylogeny with activity of cells (Figure 22.27)

As the fluid stream exits the nozzle, it is broken into droplets containing no more than a single cell. Droplets containing desired cell types (detected by fluorescence or light scatter) are charged and collected by redirection into collection tubes by positively or negatively charged deflection plates.
An uncultured filamentous cell belonging to the *Gammaproteobacteria* is shown to be an autotroph (as revealed by MAR-measured uptake of $^{14}\text{CO}_2$).

Uptake of $^{14}\text{C}$-glucose by a mixed culture of *Escherichia coli* (yellow cells) and *Herpetosiphon aurantiacus* (filamentous, green cells).

MAR of the same field of cells shown in (b). Incorporated radioactivity exposes the film and shows that glucose was assimilated mainly by cells of *E. coli*. 