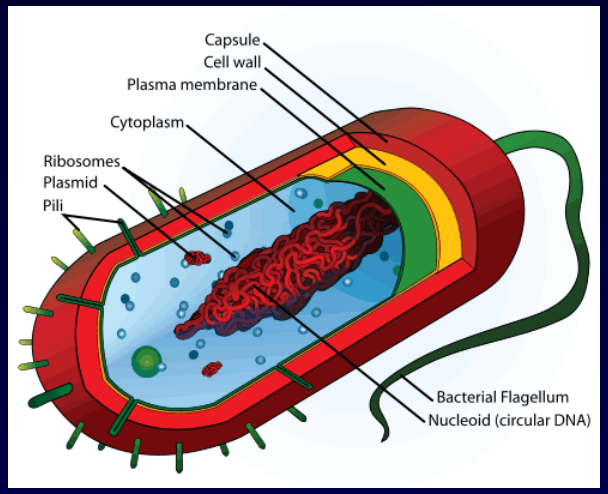
Microbiological methods

Dr. Lidita Khandeparker

What are Bacteria??



A typical bacterium

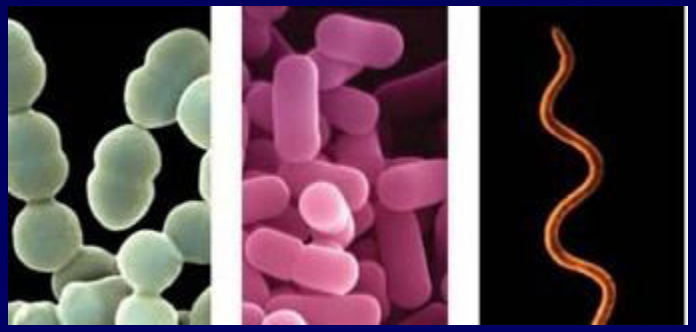
✓ Bacteria are a group of single-celled microorganisms with prokaryotic (an organism without a nucleus) cellular configuration

✓ The organelles of eukaryotes (mitochondria and chloroplasts) are thought to be remnants of Bacteria that invaded, or were captured by primitive eukaryotes in the evolutionary past

✓ Numerous types of eukaryotic cells that exist today are inhabited by endosymbiotic prokaryotes

✓ A typical bacterial cell is about 1 micrometer in diameter or width

Bacteria occur in three main shapes



Spherical **Rod shaped** **Spiral**

How they have evolved??

Modern bacteria's ancestors - single-celled microorganisms - appeared on earth about 4 billion years ago. They were the first life forms on Earth.

✓ All prokaryotes were once placed in the kingdom Monera

✓ Recently, they are divided into two different kingdoms: the Eubacteria and the Archaebacteria

Archaebacteria

- Very old bacteria, evolved a long time ago
- Many live in extreme environments such as hot springs, salty water and acidic environments
- Some live in or on organisms
- Autotrophic or heterotrophic
- Can be harmful or helpful

Eubacteria

- More recently evolved bacteria
- Live in or on organisms
- Autotrophic or heterotrophic
- Can be harmful or helpful

How do bacteria feed themselves?

Heterotrophic bacteria

Autotrophic bacteria

- Photoautotrophs
- Chemoautotrophs

Nutritional Type	Energy Source	Carbon Source	Examples
Photoautotrophs	Light	CO_2	Cyanobacteria, some Purple and Green Bacteria
Photoheterotrophs	Light	Organic compounds	Some Purple and Green Bacteria
Chemoautotrophs or Lithotrophs (Lithoautotrophs)	Inorganic compounds, e.g. H_2, NH_3, NO_2, H_2S	CO_2	A few Bacteria and many Archaea
Chemoheterotrophs or Heterotrophs	Organic compounds	Organic compounds	Most Bacteria, some Archaea

How do **bacteria** multiply ?

- Bacteria can reproduce at tremendous speeds
- Some bacteria can reproduce as often as once every 20 minutes!
- However, bacteria have to have certain conditions in which to reproduce
- These conditions are not often met, and that is one thing that keeps bacteria from growing out of control

Bacteria reproduce using two basic methods

Asexual reproduction involves only one individual or parent. The offspring generated by asexual reproduction are exact duplicates of the parent.
Binary fission is the process by which a bacteria splits into two cells. Each cell gets an exact copy of the parent cell's genetic material.

Sexual reproduction involves joining of two parent cells and exchanging of genetic materials. The offspring will have a mixture of parent cells' traits. **Conjugation** is the process by which bacteria join and exchange genetic materials. Once genetic materials are exchanged, each bacteria cell will go through binary fission to produce an offspring with a new genetic makeup

How they are unique?

Bacteria inhabit different kinds of environments

Aerobes (aerobic bacteria)

Anaerobes (anaerobic bacteria)

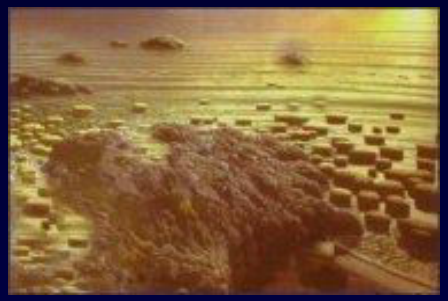
Facultative anaerobes (facultative anaerobic bacteria)

Mesophile (mesophilic bacteria)

Extremophiles (extremophilic bacteria)

- Thermophiles (thermophilic bacteria)
- Halophiles (halophilic bacteria)
- Acidophiles (acidophilic bacteria)
- Alkaliphiles (alkiliphilic bacteria)
- Psychrophiles (psychrophilic bacteria)

Bacteria are found everywhere



- Soil
- Radioactive waste
- Water
- Plants
- Animals
- Deep in the earth's crust
- Organic material
- Arctic ice
- Glaciers
- Hot springs
- The stratosphere (between 6 to 30 miles up in the atmosphere)
- Ocean depths - they have been found deep in ocean canyons and trenches over 32,800 feet (10,000 meters) deep.
- They live in total darkness by thermal vents at incredible pressure.

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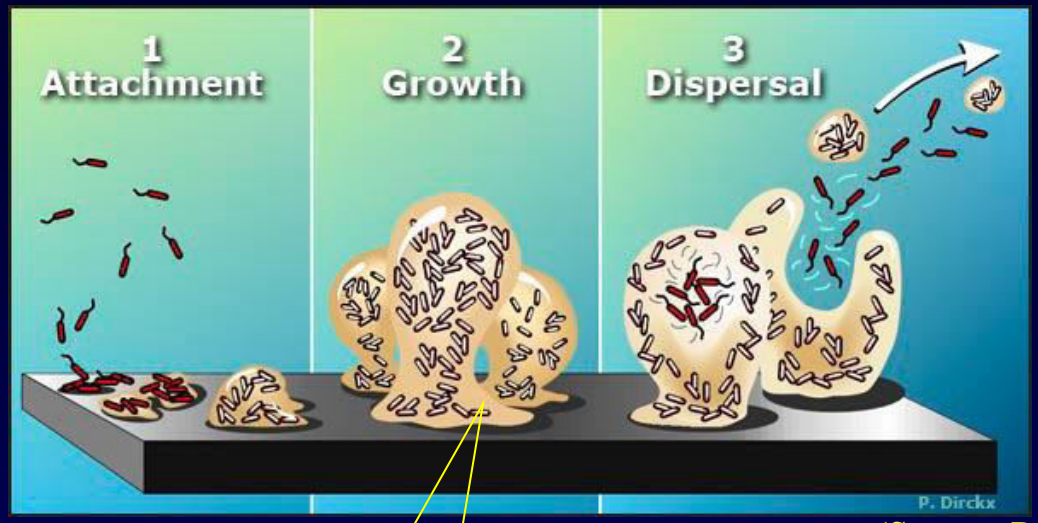
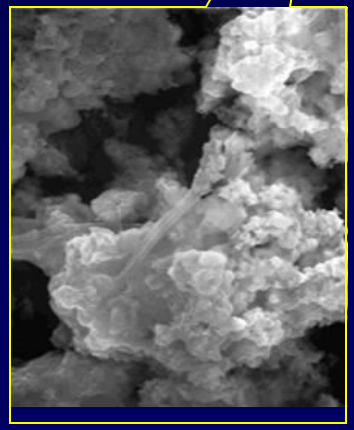
understanding the seas

How bacteria colonize a surface?



(Source: P. Dirckx)



Biofilm

Phases of Bacterial growth

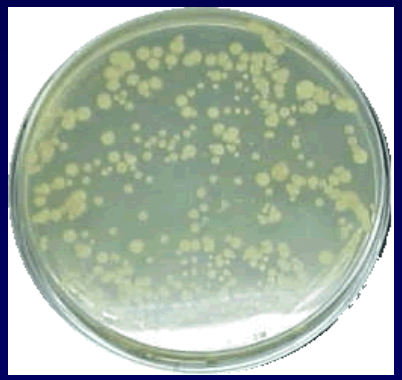
1) Lag phase
2) Log (logarithmic) phase
3) Stationary phase
4) Decline phase or death phase

Growth in Colonies

A pure culture contains only one species or strain

A colony is a population of cells that arise from a single cell or spore or from a group of attached cells

A colony is often called as a colony-forming unit (CFU)



Bacterial Quantification

Direct methods
Plate counts
MPN
Direct microscopic count/Flow cytometry
Dry weight

Indirect methods
Turbidity
Metabolic activity
Dry weight

Methods to classify Bacteria

Phenotypic Classification
Microscopic morphology
Macroscopic morphology
Biotyping
Serotyping
Antibiogram patterns
Phage typing

Genotypic Classification
Guanine and cytosine ratio
DNA hybridization
Nucleic acid sequence analysis
Plasmid analysis
Ribotyping
Chromosomal DNA fragment

Analytic Classification
Whole cell lipid analysis
Whole cell protein analysis
Multifocus locus enzyme electrophoresis
Cell wall fatty acid analysis

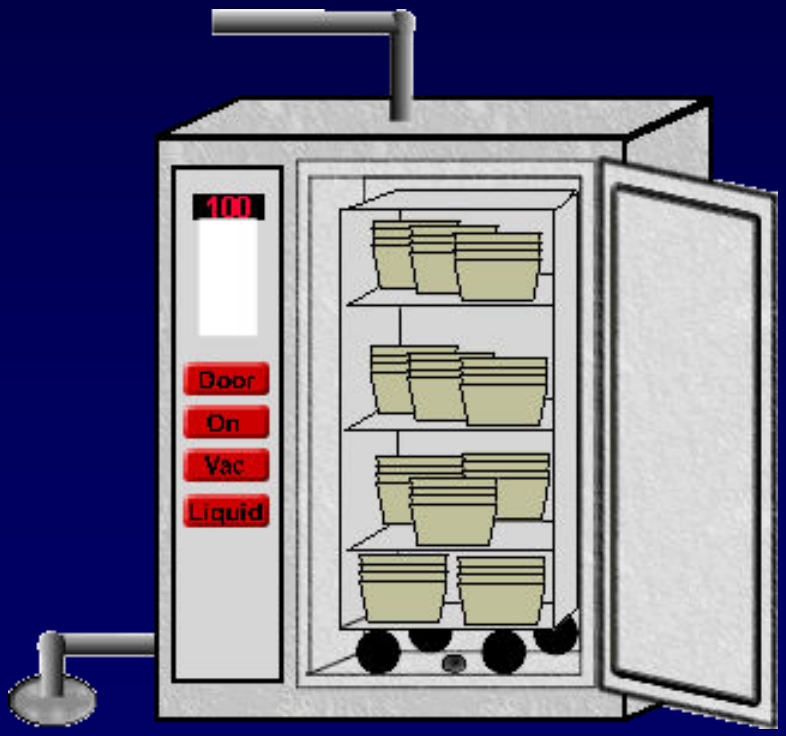
Bacterial Identification

- The **Gram stain**, which divides most bacteria into two main groups, is the first step in bacterial identification.
- Cell shape and size
- Acid fast reaction
- Special structures

Sterilization

Moist heat, dry heat, chemicals, and radiation

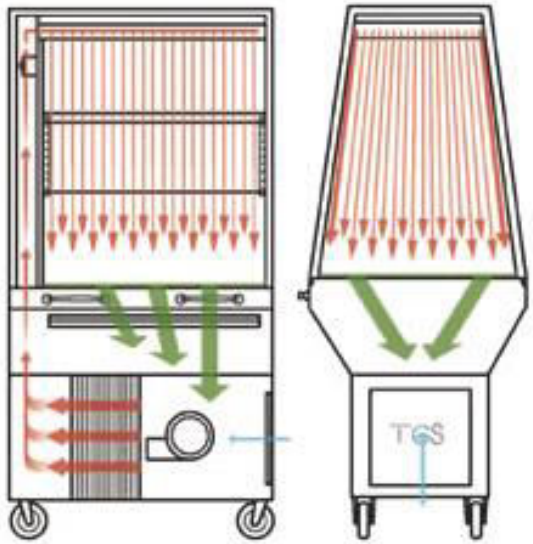
- Steam autoclave is primary means of sterilizing



Autoclave

Steam in at top, displaces air out drain in bottom.
Load the equipments to be sterilized.
To start, close door tightly and turn on timer.
When the temperature reaches 121°C and pressure reaches 15 pounds per square inch (psi), the timer begins sterilization time.
Steam is then vented.

Contd..



Thoren Caging uses a different air flow as well.

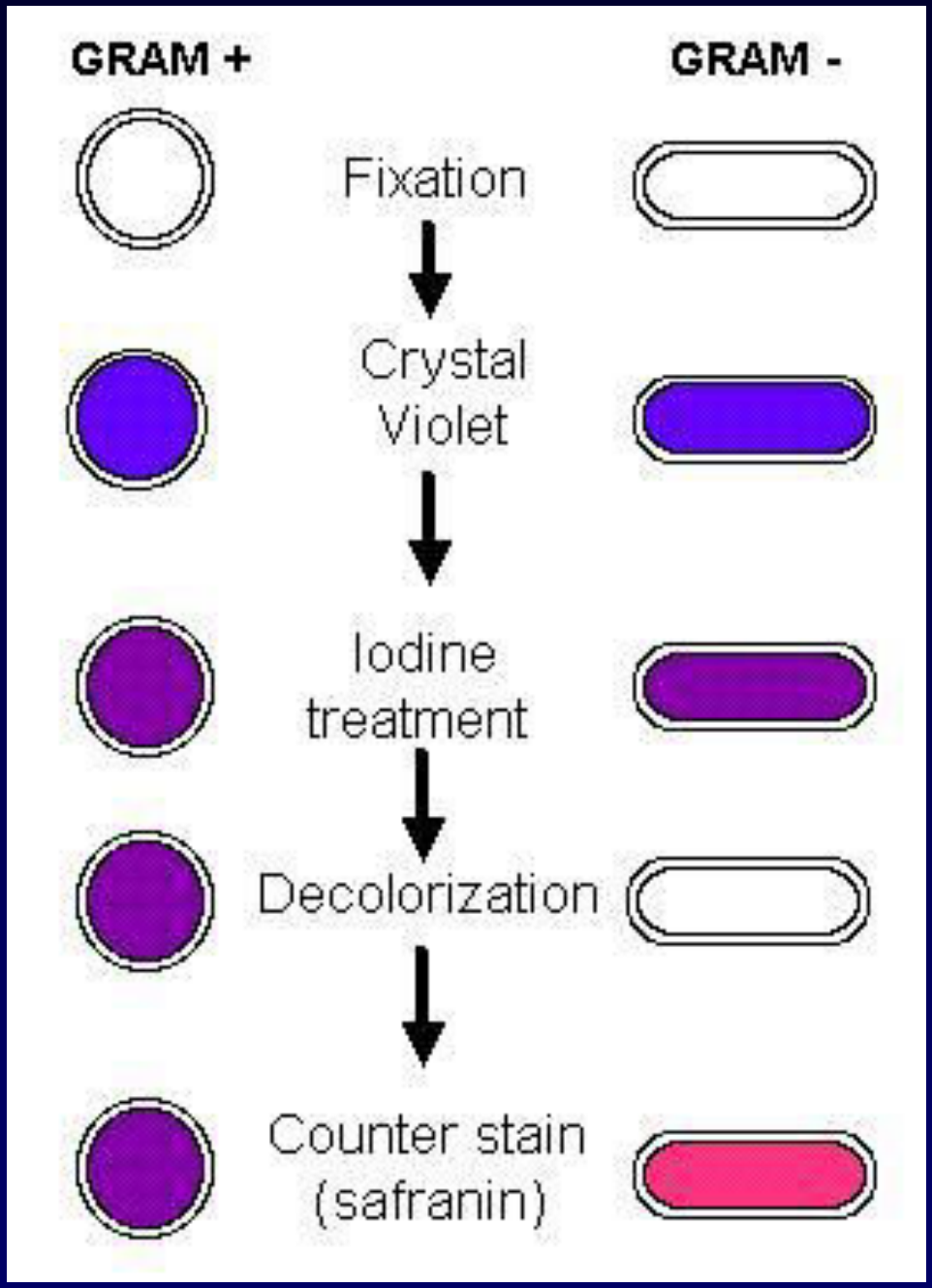
(Click on the picture to view the web page.)

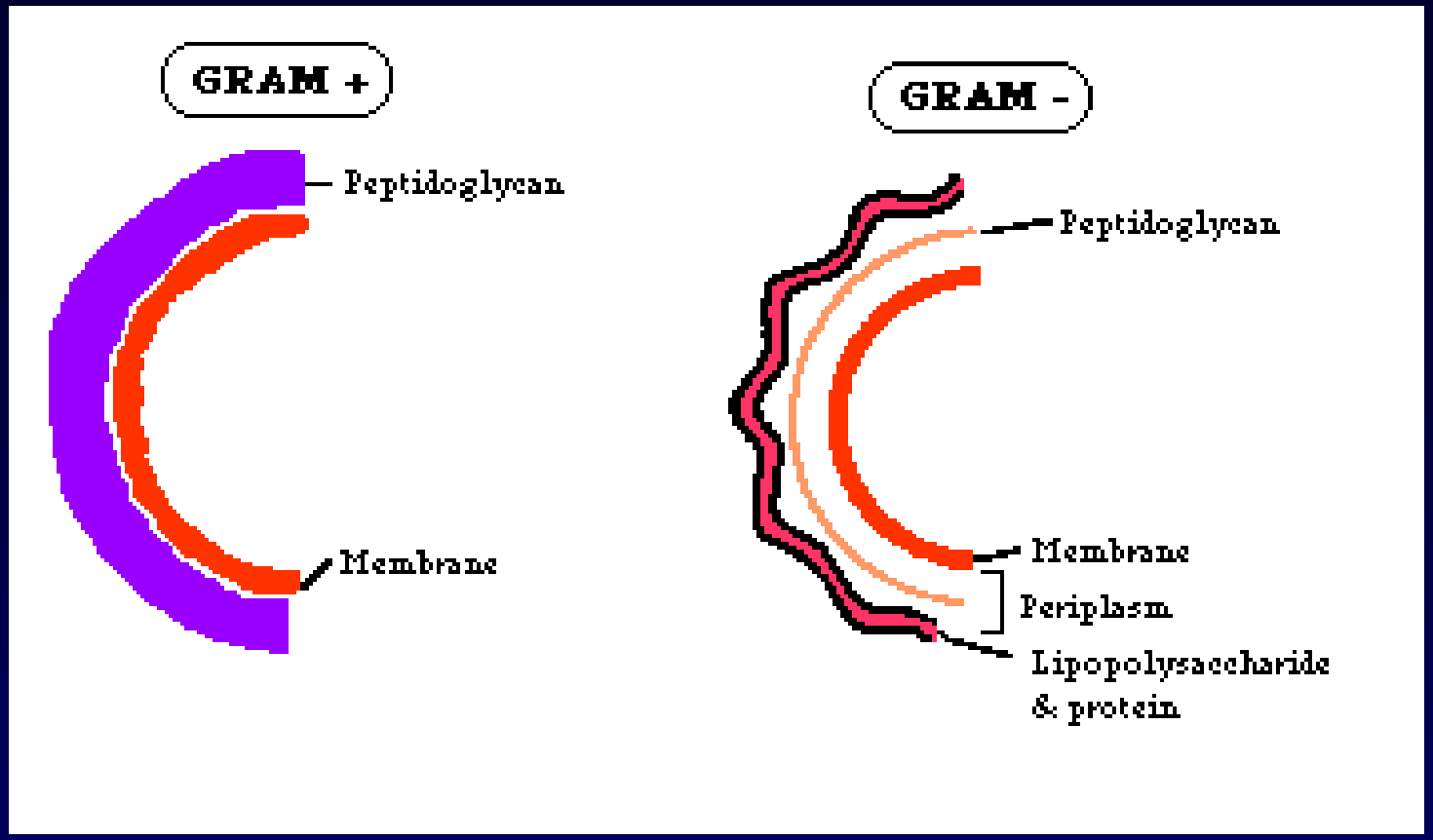
Courtesy of Thoren Caging (www.thoren.com)

Laminar flow

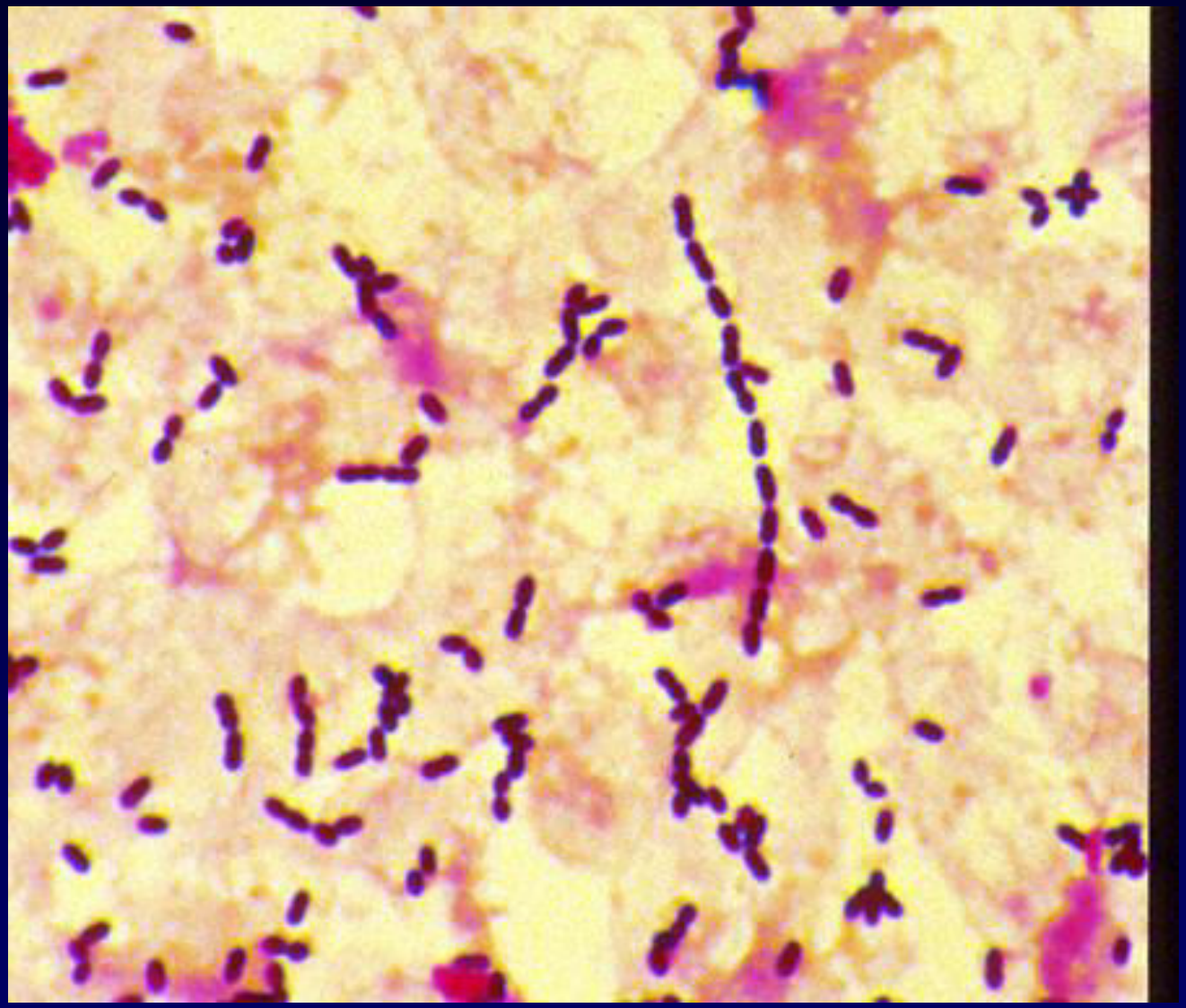
Physiological/Biochemical Characteristics

- Traditional mainstay of bacterial identification
- Diagnostic tests for determining the presence of specific enzymes and assessing nutritional and metabolic activities
- Examples
  - Fermentation of sugars
  - Capacity to metabolize complex polymers
  - Production of gas
  - Presence of enzymes
  - Sensitivity to antimicrobial drugs

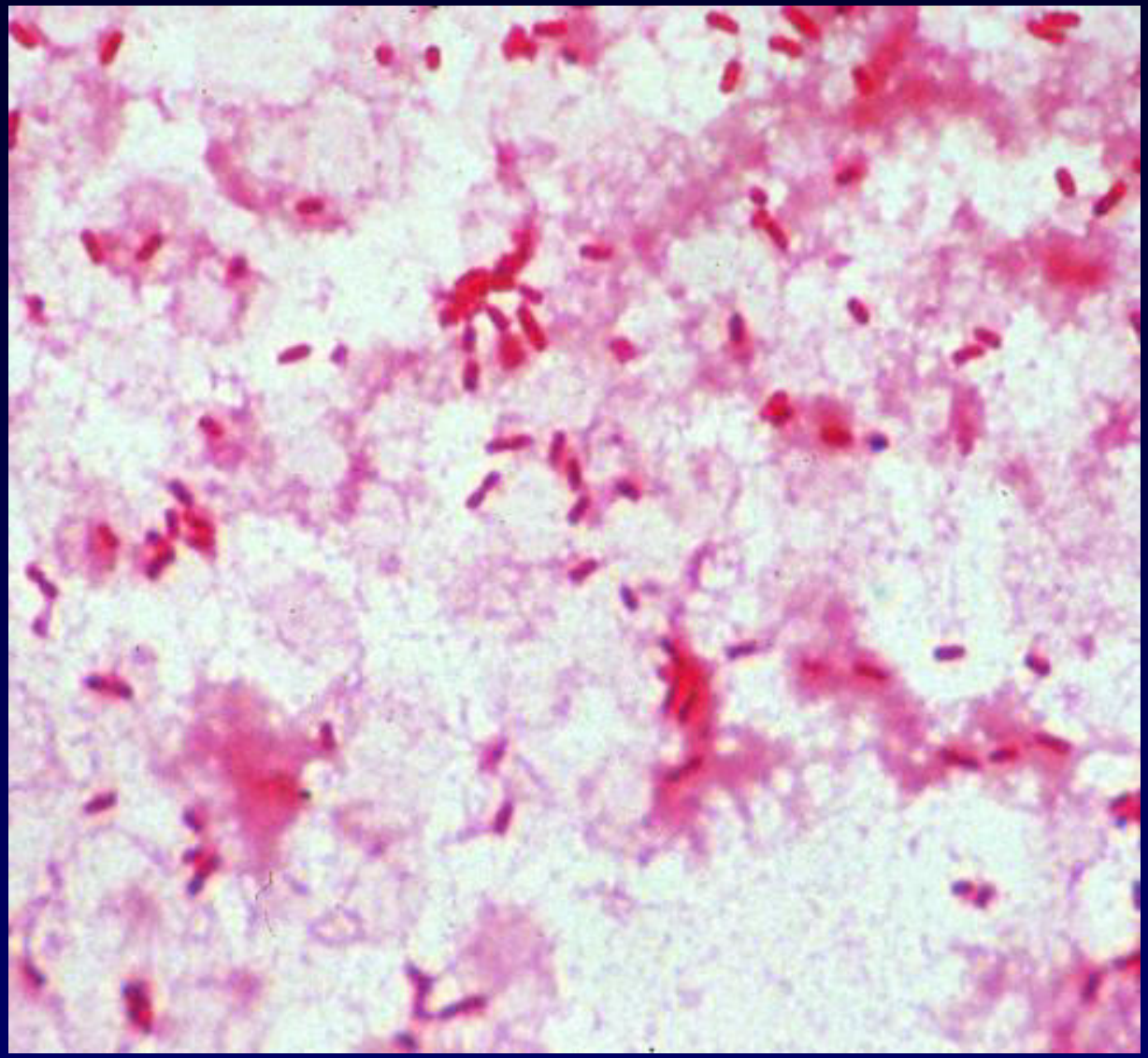




In **Gram-positive bacteria, the purple crystal violet stain is trapped by the layer of peptidoglycan** which forms the outer layer of the cell. In **Gram-negative bacteria, the outer membrane of lipopolysaccharides prevents the stain from reaching the peptidoglycan layer**. The outer membrane is then permeabilized by acetone treatment, and the **pink safranin counterstain** is trapped by the peptidoglycan layer.



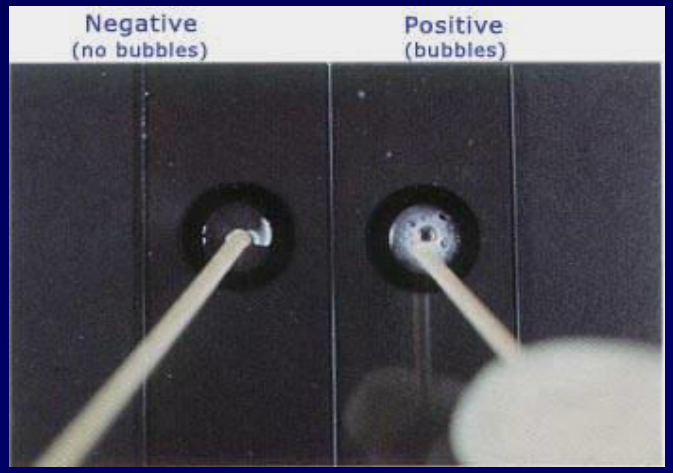
Is this gram stain positive or negative?
Identify the bacteria.



Is this gram stain positive or negative?
Identify the bacteria.

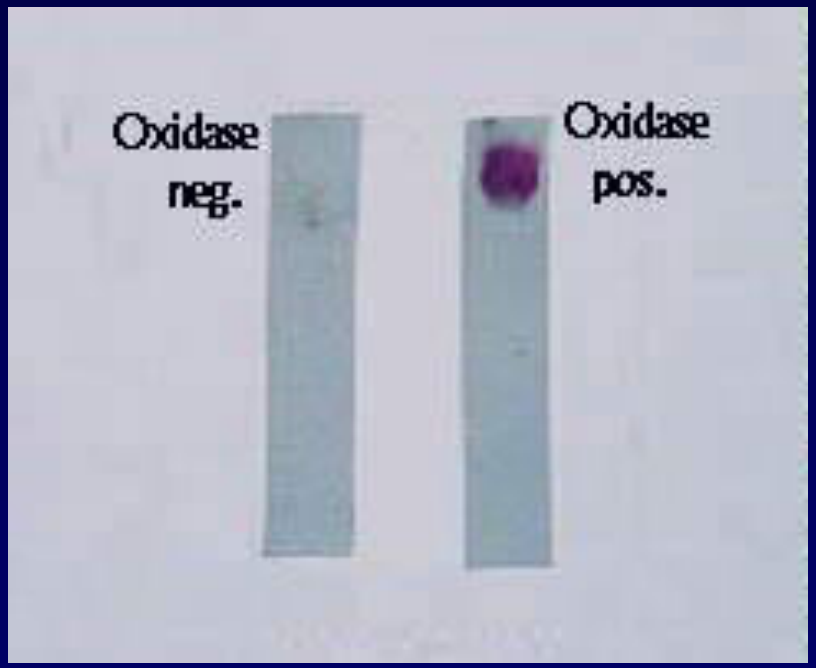
Catalase test

- This test is used to differentiate those bacteria that produce the enzyme catalase, such as *staphylococci*, from non-catalase producing bacteria such as *streptococci*.
- This enzyme converts hydrogen peroxide into water and oxygen.



Oxidase Test

When the organism is oxidase-producing, the phenylenediamine (oxidase reagent) will be oxidized to a deep purple color.



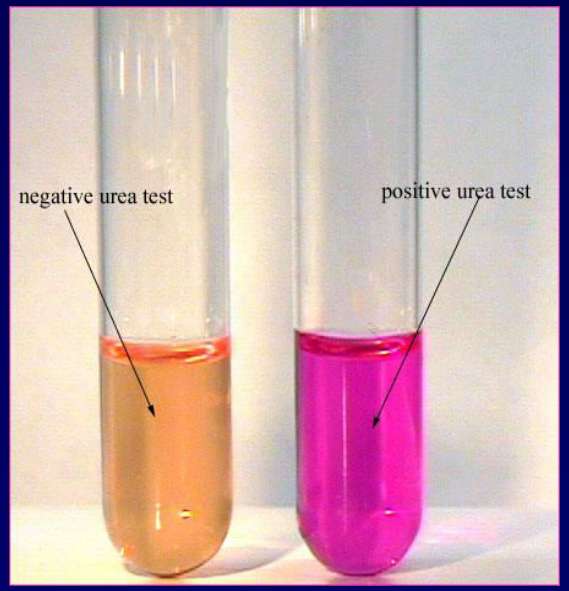
Urease Test

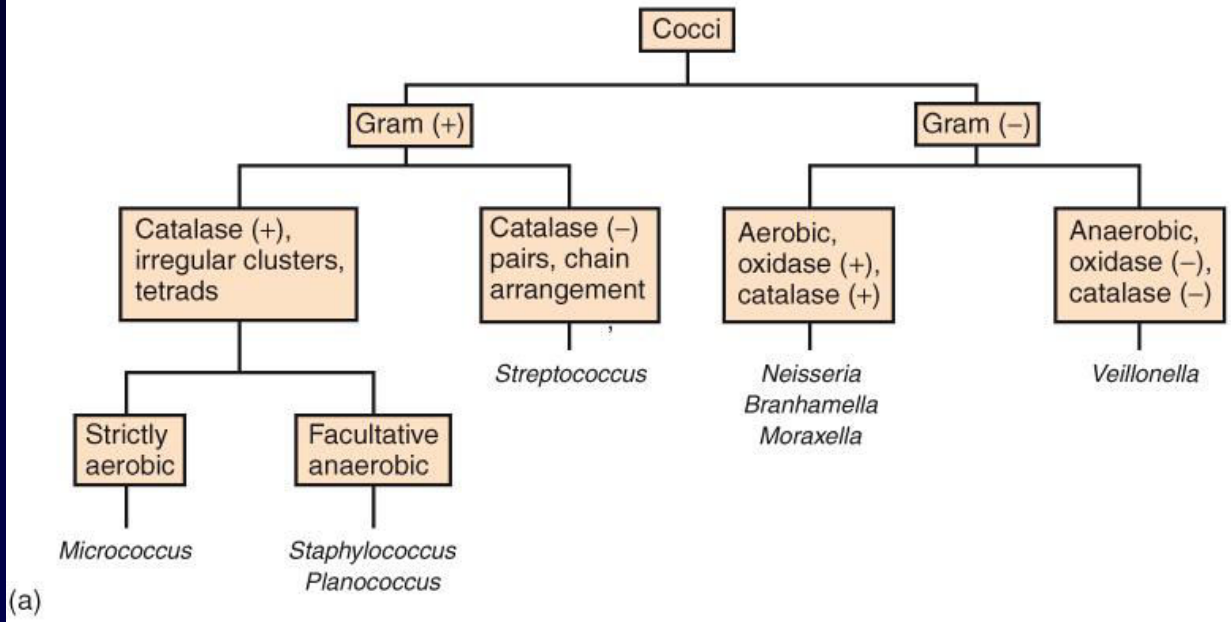
Proteus strains are strong urease producers.

Principle:

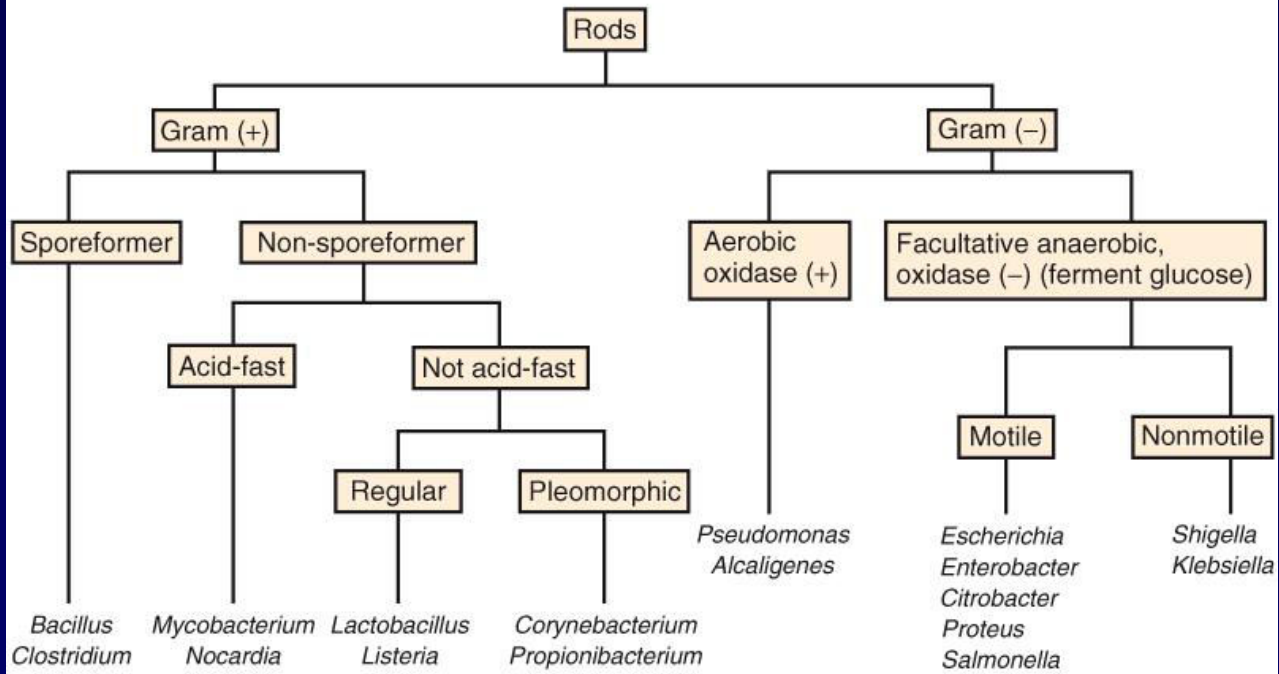
The test organism is cultured in a medium which contains urea and the indicator phenol red. When the strain is urease producing, the enzyme will break down the urea to give ammonia and carbon dioxide.

With the release of ammonia, the medium becomes alkaline as shown by a change in colour of the indicator to pink-red.





(a)



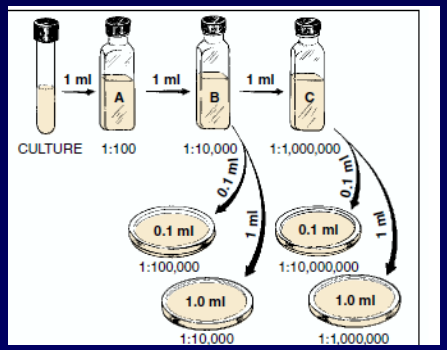
(b)

Protocols For Viable Bacterial Enumeration



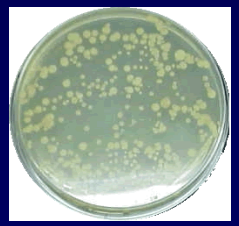

Pouring Plate using selective media

Processing of Samples for viable bacteria



Serial Dilutions

Spreading inoculum on surface evenly



Colonies grow

Count colonies

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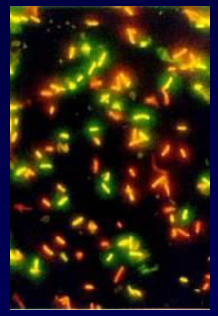
Bacterial abundance - Total Bacterial Count (TBC) using epifluorescence microscopy

The two most widely used fluorochromes used to identify bacteria are

- Acridine Orange (AO) – 3,6-bis[dimethylamino]acridinium chloride
- DAPI - 4,6-diamidino-2-phenylindole

(AODC method)

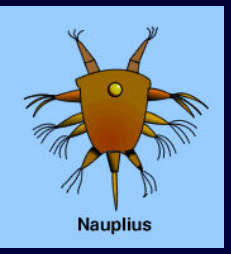


➢ Acridine Orange binds to both DNA and RNA with an excitation maximum of approximately 470 nm

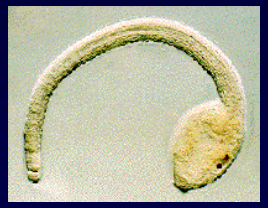
➢ AO-stained single-stranded nucleic acids emit orange-red fluorescence, while those that are double stranded fluoresce green in vivo

The distribution of dead, metabolically inactive but alive and living cells cannot be determined as DNA retains its staining properties even in non-viable cells.

What is Viability of organisms



Living



Dead?

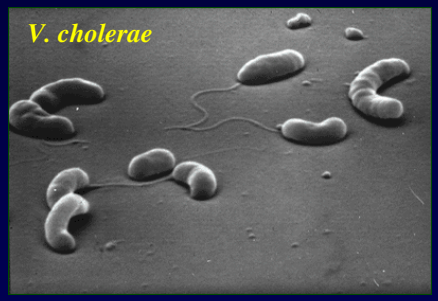
Viable?

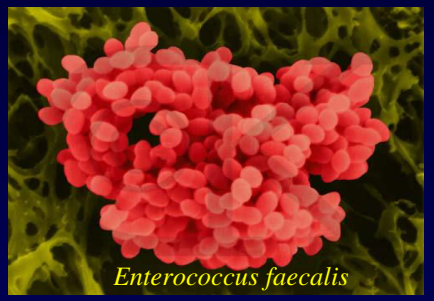
Two color assays

- ✓ Epifluorescence microscopy
- ✓ Confocal microscopy
- ✓ Flowcytometry

Flow cytometry allows rapid enumeration, physical and biochemical characterization of cells from a non-homogenous population

Important Pathogenic Bacteria





Protocols For Bacterial Enumeration

Collection of Samples

Surface

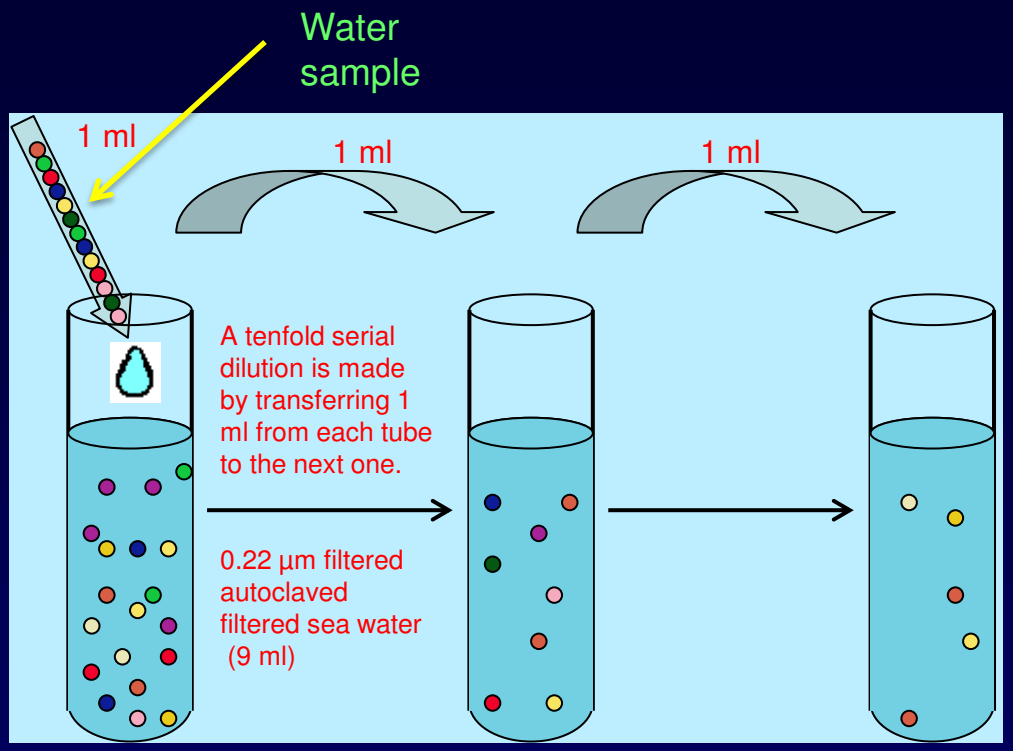
Bottom

Zooplankton

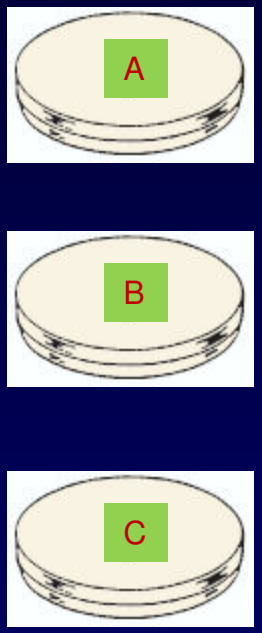
Sediment

Fouling

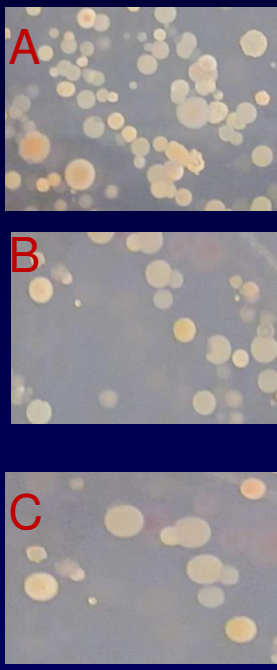
Protocol – water sample



Dispense 0.1 ml



Count the colonies



A (10^{-1}) B (10^{-2}) C (10^{-3})

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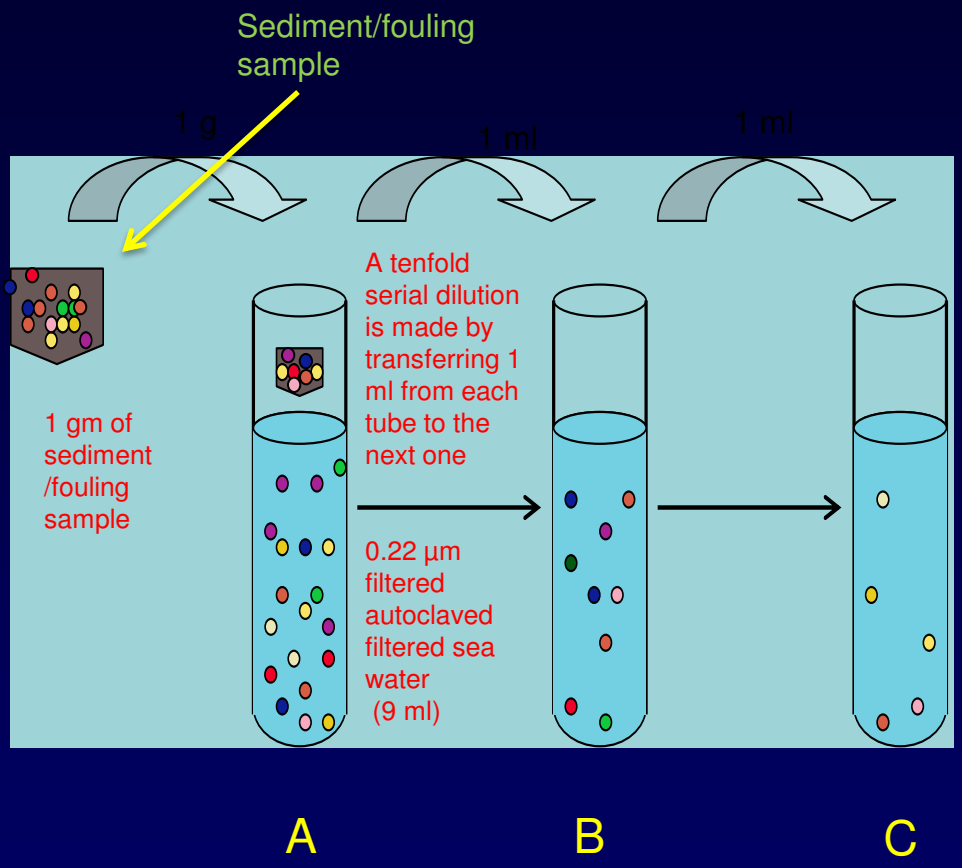
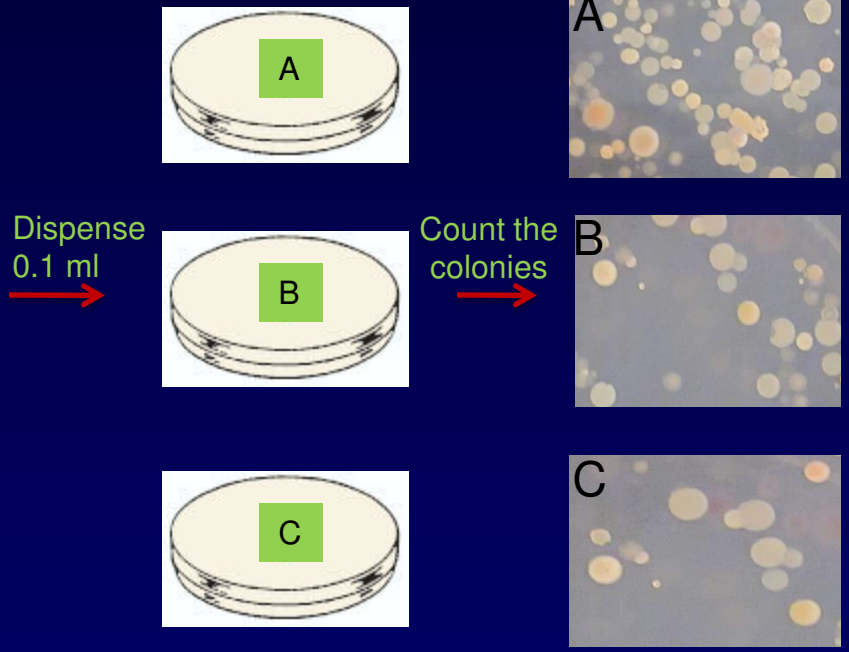
Protocol – Sediment/fouling sample





Protocol – Zooplankton sample

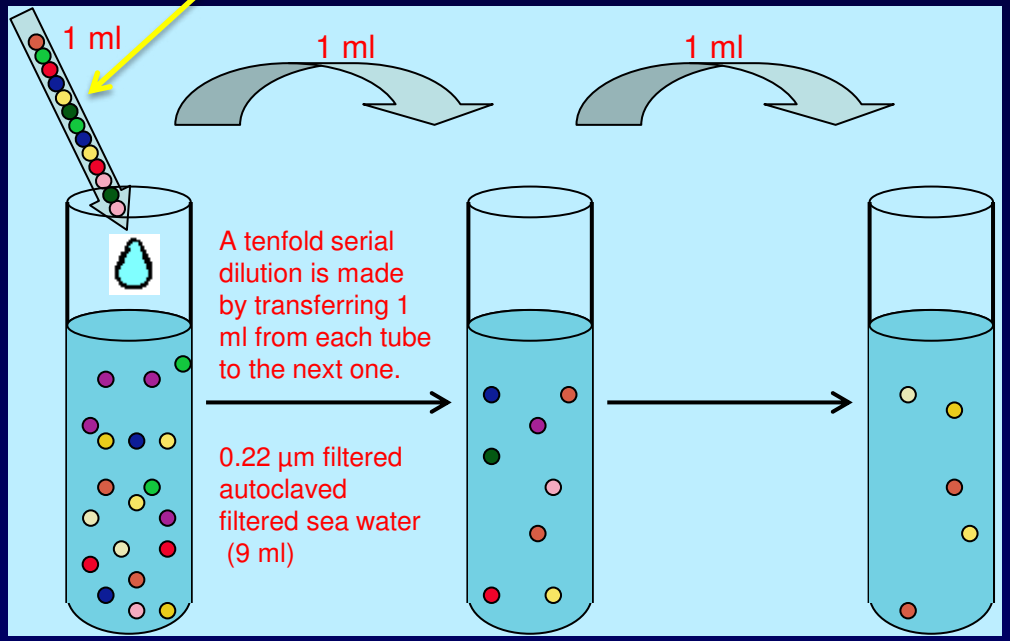
Sampling Net

Filter through 50µm mesh

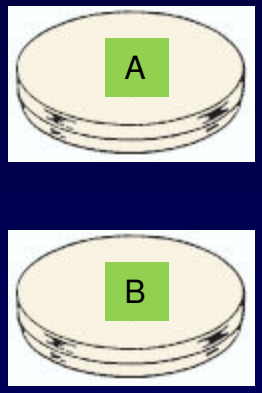
Zooplankton

Homogenize the sample

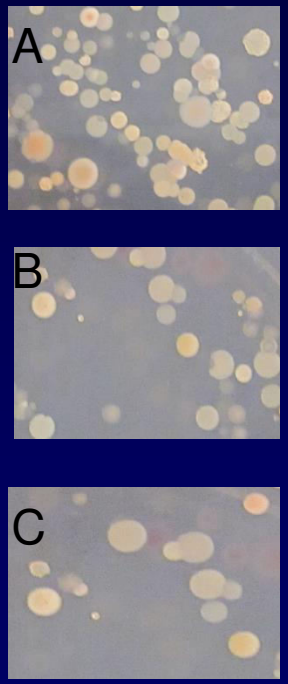
Homogenized sample



Dispense 0.1 ml



Count the colonies



A (10^{-1}) B (10^{-2}) C (10^{-3})

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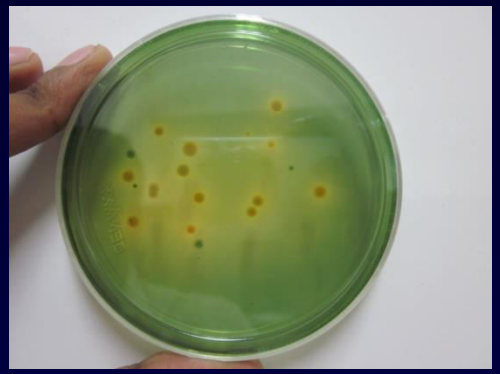
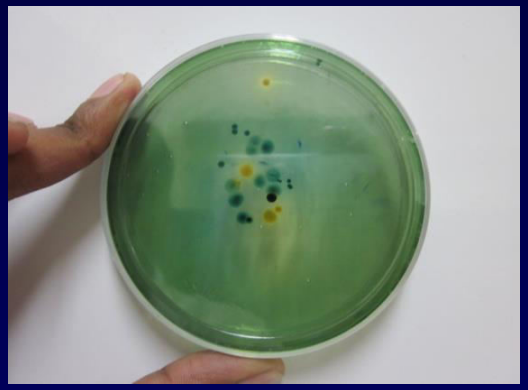
Enumeration of Total Viable Pathogenic bacterial abundance using different selective media

Medium	Bacteria	Observation
Thiosulfate Citrate Bile Sucrose Agar (TCBS)	*Vibrio cholerae*	Yellow colored ones, <2mm
	Vibrio alginolyticus	Yellow colored ones, >2mm
	Vibrio parahaemolyticus	Green color colonies
	Aeromonas/Pseudomonas spp.	Blue color colonies
MacConkey Agar	*Escherichia coli*	Pink color colonies
	Salmonella/Shigella	White color colonies
Enterococcus confirmatory Agar	*Streptococcus faecalis/ Enterococcus faecalis*	Blue color colonies
	General Streptococci	White color colonies
E.coli 0157 H:7 Agar	*Escherichia coli* 0157	Purple color colonies
	Salmonella serotype Enteritidis	White color colonies
Xylose Lysine deoxycholate Agar (XLD)	*Salmonella* spp.	Pink color with Black centered colonies
	Shigella spp.	Pink color colonies

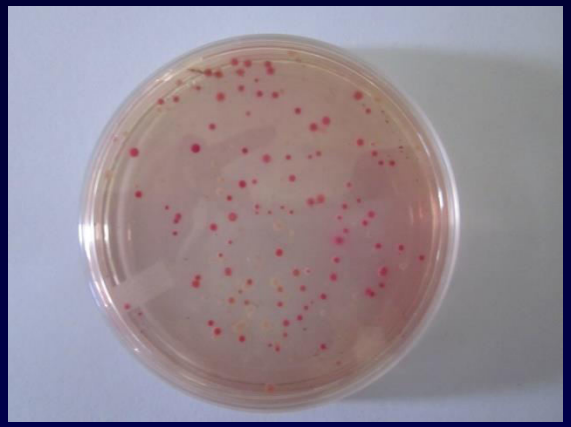
Thiosulfate Citrate Bile Sucrose Agar (TCBS)

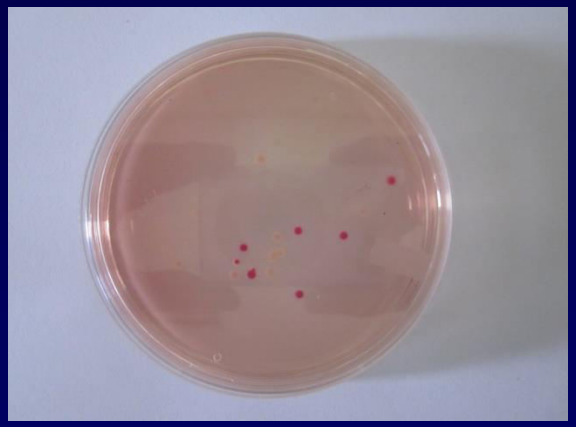




Thiosulfate Citrate Bile Sucrose Agar (TCBS)	***Vibrio cholerae***	**Yellow colored ones, <2mm**
	Vibrio alginolyticus	**Yellow colored ones, >2mm**
	Vibrio parahaemolyticus	**Green color colonies**
	Aeromonas/Pseudomonas **spp**	**Blue color colonies**

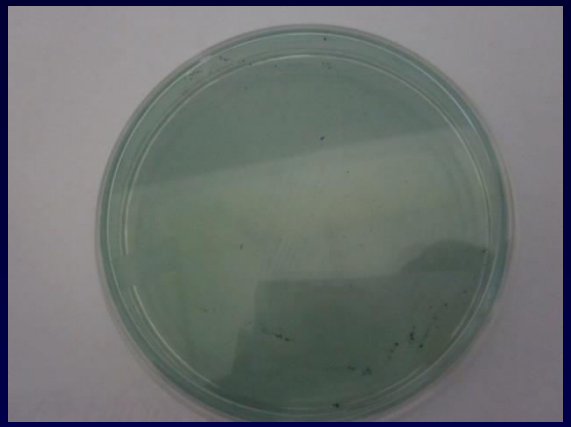
MacConkey Agar





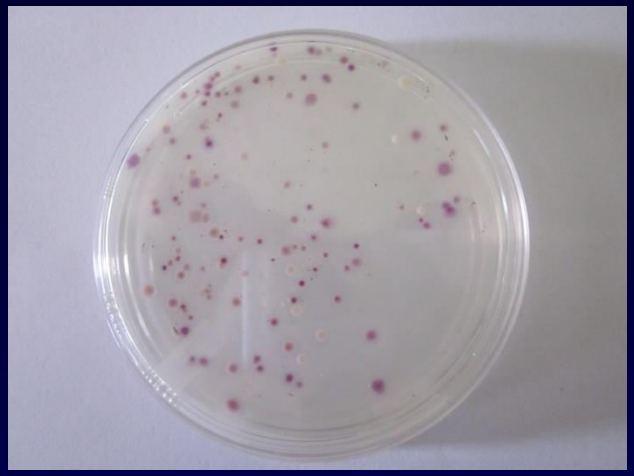
MacConkey Agar	***Escherichia coli***	**Pink color colonies**
	Salmonella/Shigella	**White color colonies**

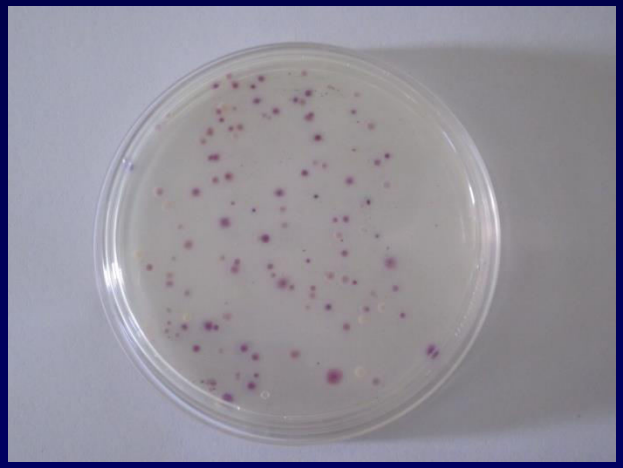
Enterococcus confirmatory Agar



Enterococcus **confirmatory Agar**	***Streptococcus faecalis/ Enterococcus faecalis***	**Blue color colonies**
	General Streptococci	**White color colonies**

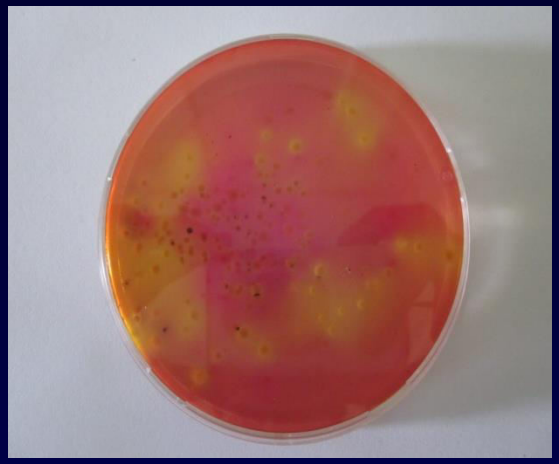
E.coli 0157 H:7 Agar

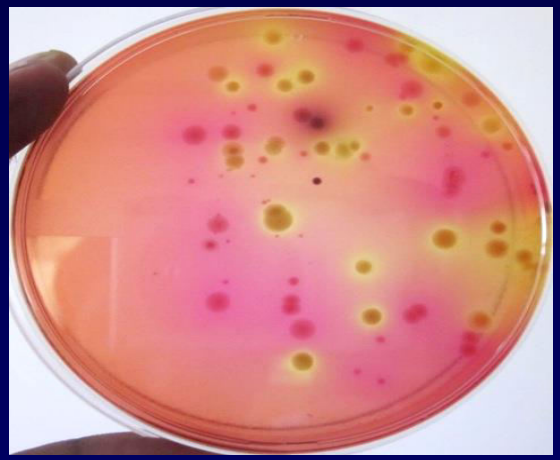




***E. coli* 0157 H:7 Agar**	***Escherichia coli* 0157**	**Purple color colonies**
	***Salmonella* serotype Enteritidis**	**White color colonies**

Xylose Lysine deoxycholate Agar (XLD)





Xylose Lysine deoxycholate Agar (XLD)	***Salmonella* spp.**	**Pink color with Black centered colonies**
	***Shigella* spp.**	**Pink color colonies**

Protocol for Total Bacterial Count (TBC) using epifluorescence microscopy

Samples are immediately fixed with 0.22µm filtered Formalin or were stored in ice until fixed (250 µl of formalin is added for 5 ml of sample)

↓

1 ml of formalin fixed sample is stained with 20 µl of Acridine orange

↓

Incubate the sample for 10 min in dark

↓

Filter the sample on 0.22 µm Black Polycarbonate filter paper

↓

Place the filter paper on a clean glass slide

↓

Place a drop of oil on top of filter paper

↓

Cover this with a clean glass cover slip

↓

Observe the slide under Epi-fluorescence microscope under oil immersion

↓

Count the number of cells in each field

↓

Take a note of 10 different fields

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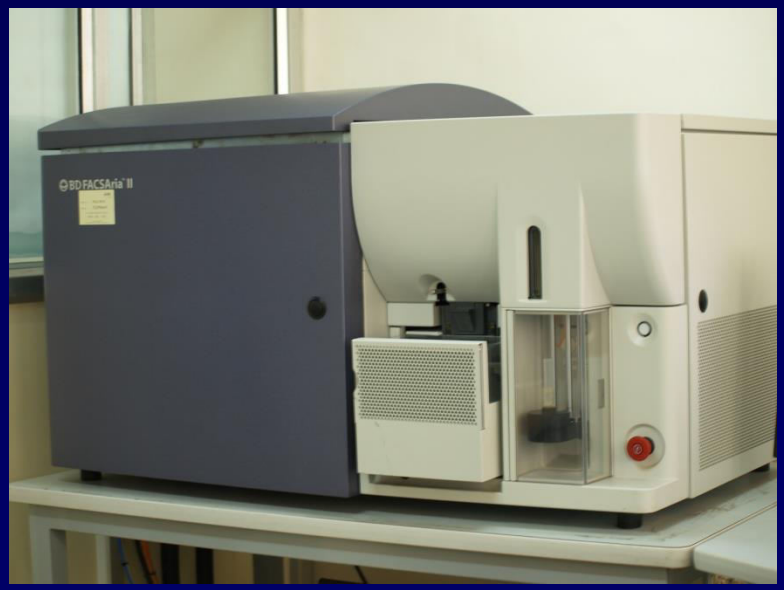
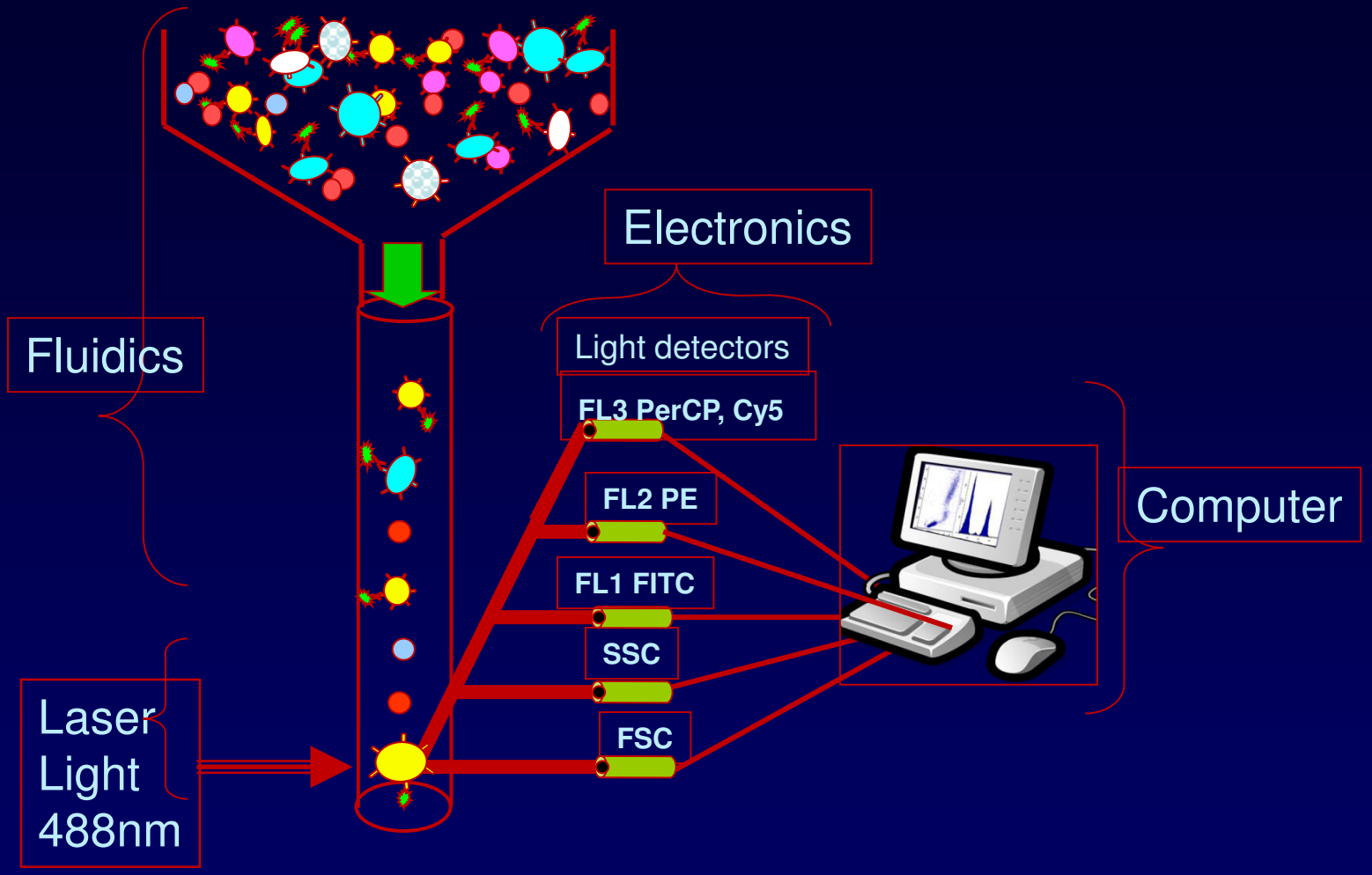
Flow cytometric assay to quantify bacteria

- ✓ Flow cytometry is a useful tool for counting microorganisms, assessing their viability and cell sorting
- ✓ Cells are usually stained or labelled with fluorescent tags that enable them to be electronically identified when passing through a beam of laser light
- ✓ When compared to conventional culturing techniques there is also the advantage of being able to detect viable but non-culturable cells in seawater and ship's ballast water which represent a major fraction of marine bacterial species

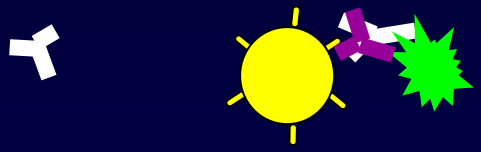


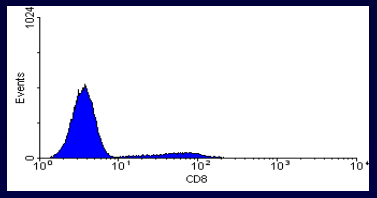
Flow cytometer consists of



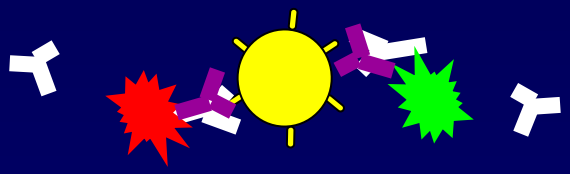
Presenting and Interpreting Data

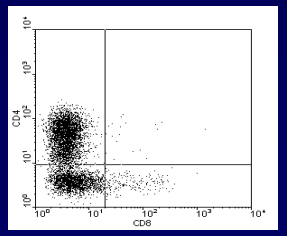
Single parameters can be displayed as a histogram.





Dual parameter data can be displayed in two dimensions using dot, density or contour plots.





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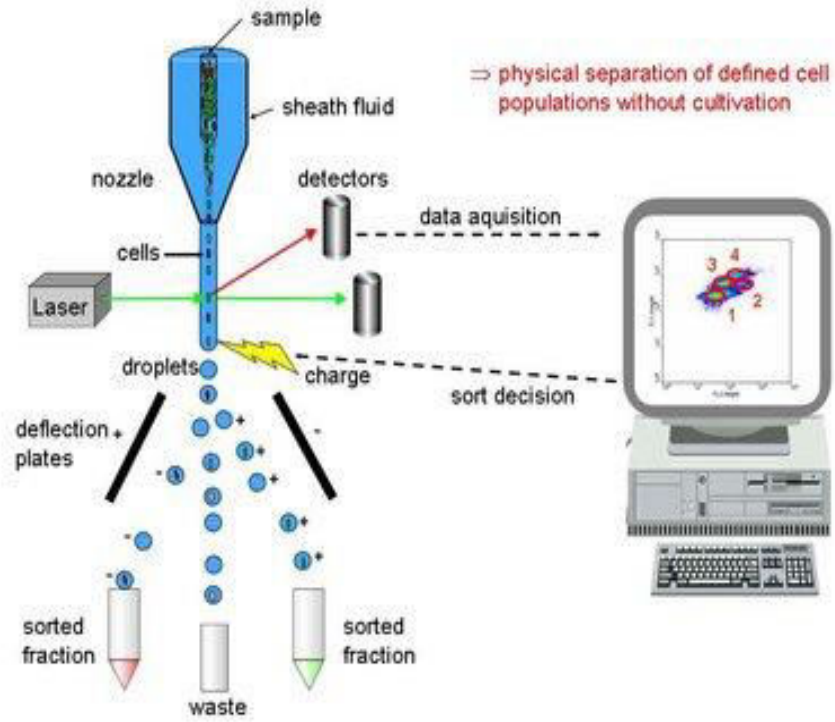
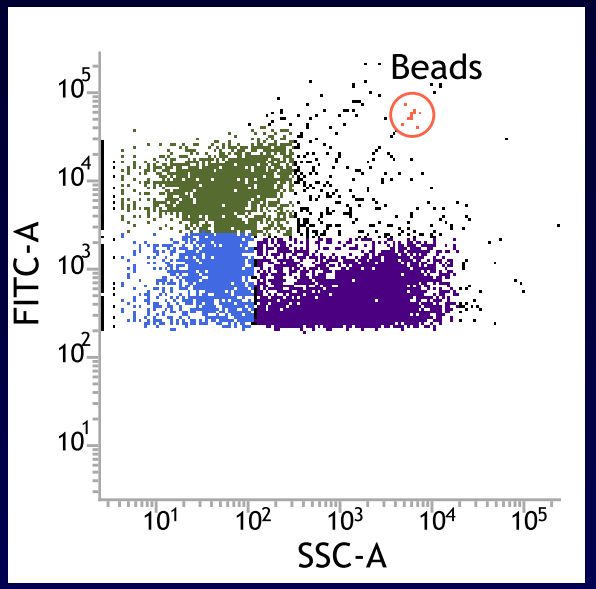
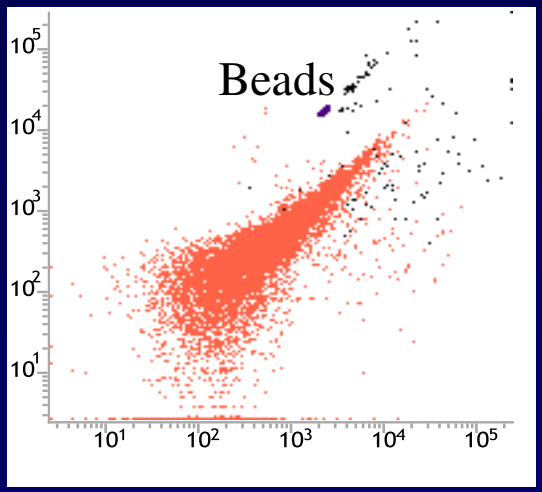
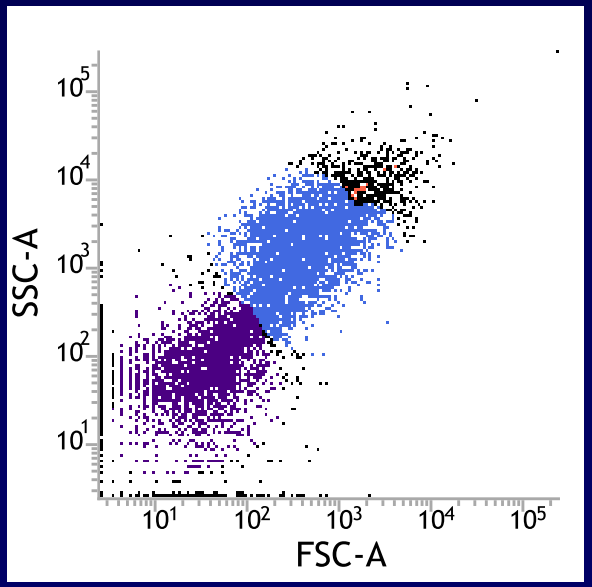




Metabolically active cells



Side scatter



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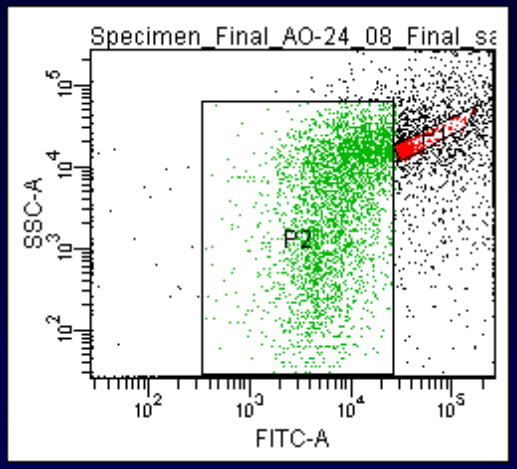
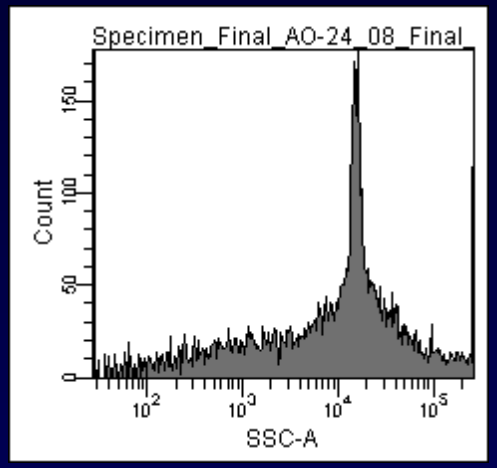
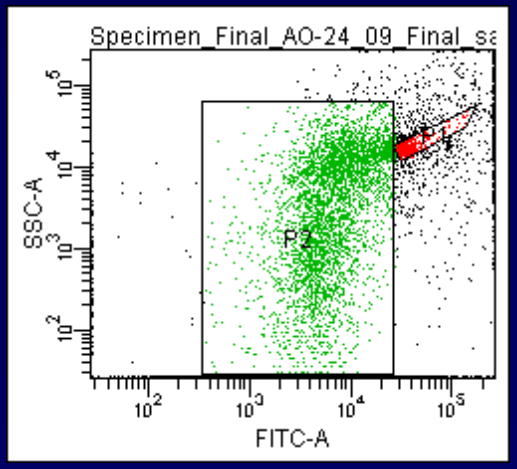
Quantification of bacteria using Acridine Orange

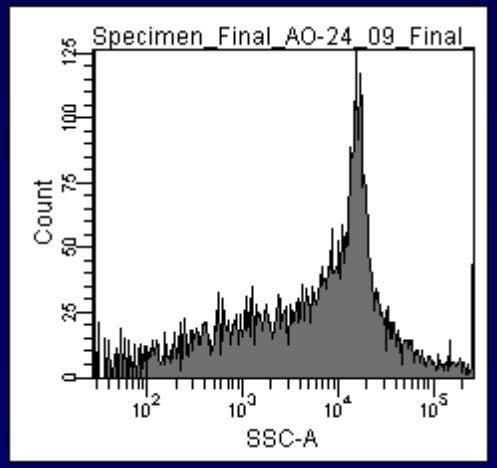




Tube: 24_08_Final_sampe_BACTERIA-AO-20µL

Population	#Events	%Parent	%Total
All Events	20,000	####	100.0
P1	766	3.8	3.8
P2	3,403	17.0	17.0





Tube: 24_09_Final_sampe_BACTERIA-AO-20µL

Population	#Events	%Parent	%Total
All Events	20,000	####	100.0
P1	496	2.5	2.5
P2	3,925	19.6	19.6

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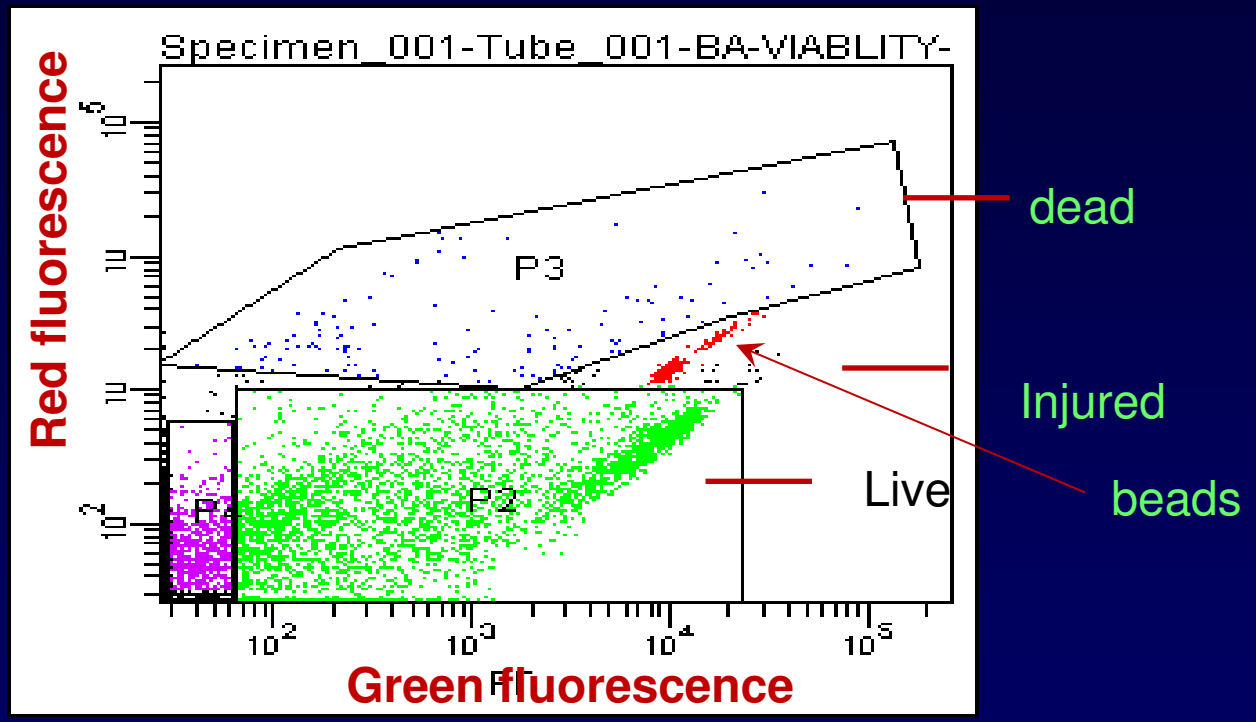
Flow cytometric assay to assess viability

Thiazole Orange

Propidium Iodide

→ To discriminate live and dead cells

Flow cytometry which offers the ability to rapidly distinguish between active and compromised or dead cells is a valuable tool to study the effect of environmental variables



Nucleic acid double staining (with SYTO9/ Propidium iodide) which differentiates active, live cells from inactive, dead cells

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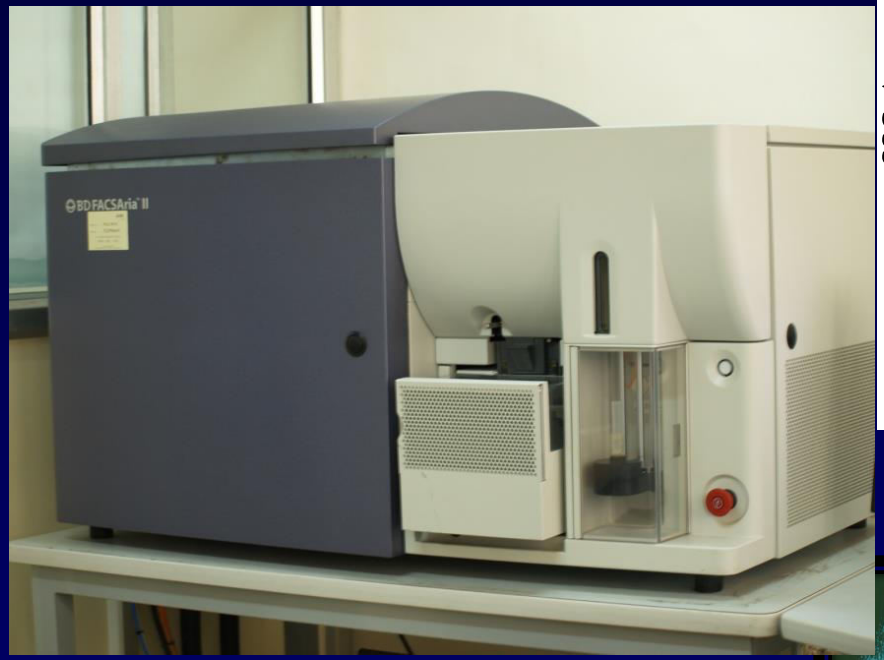
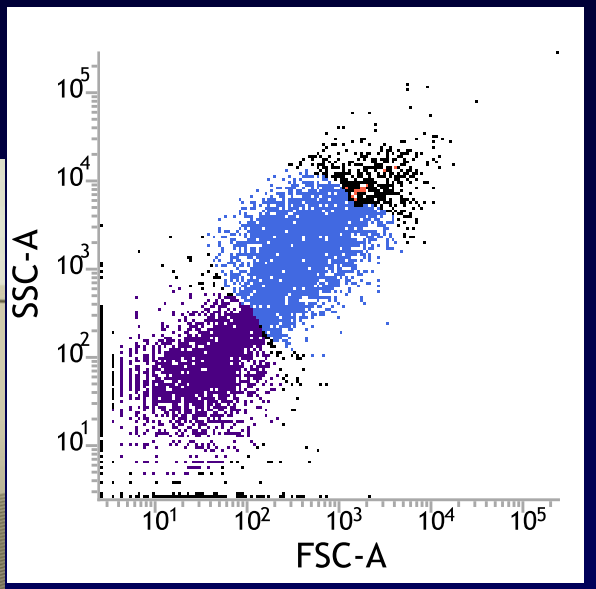
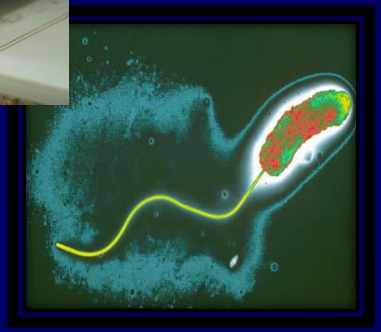
FISH-FCM method for rapid estimation of bacterial communities with special reference to *Vibrio cholerae*







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Flow cytometry with FISH

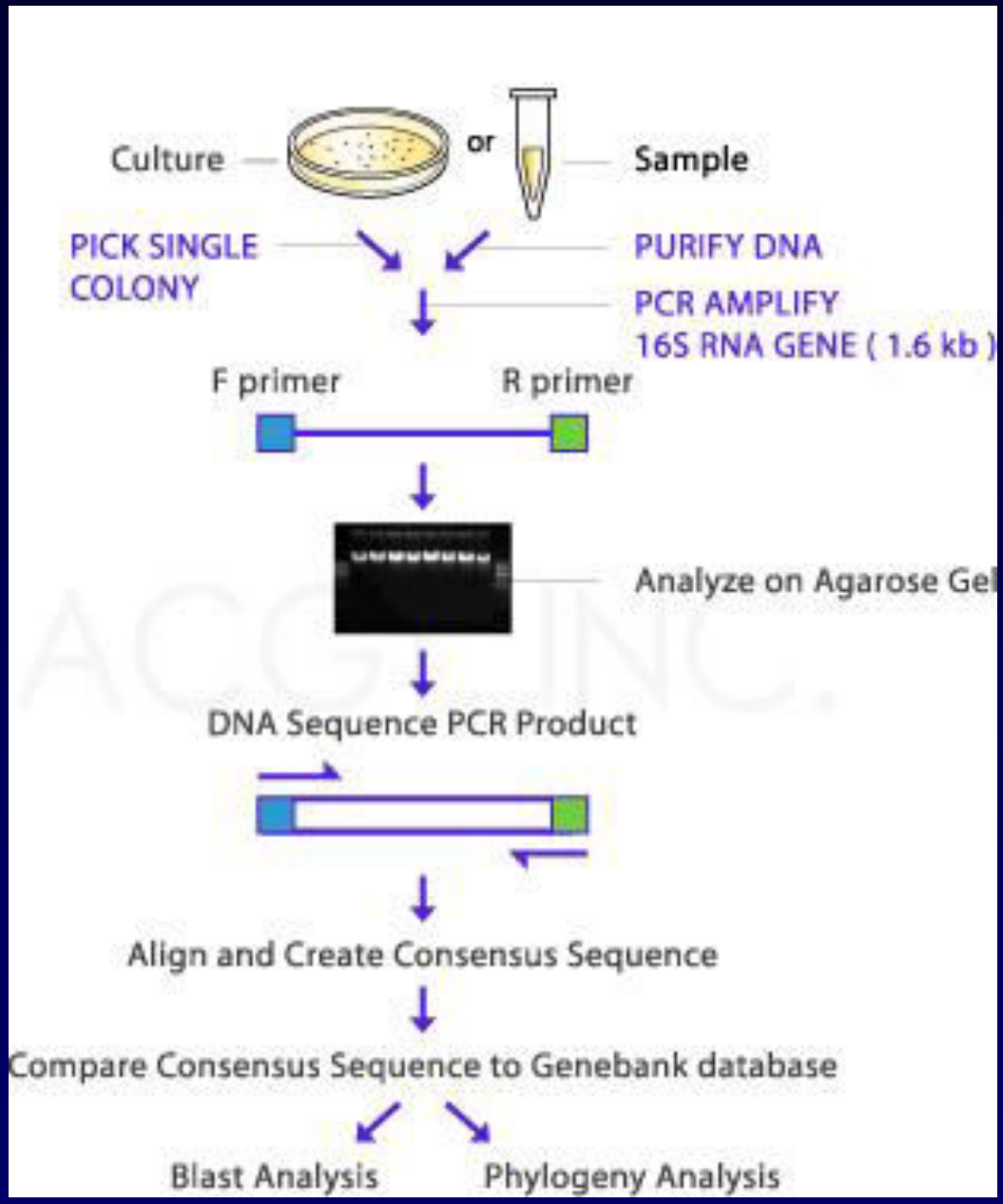
✓ Flow cytometry combined with FISH is also an increasingly popular method of enumerating cells in environmental samples

✓ The oligonucleotide probes used for FISH are dependant upon the micro-organism which is to be identified.

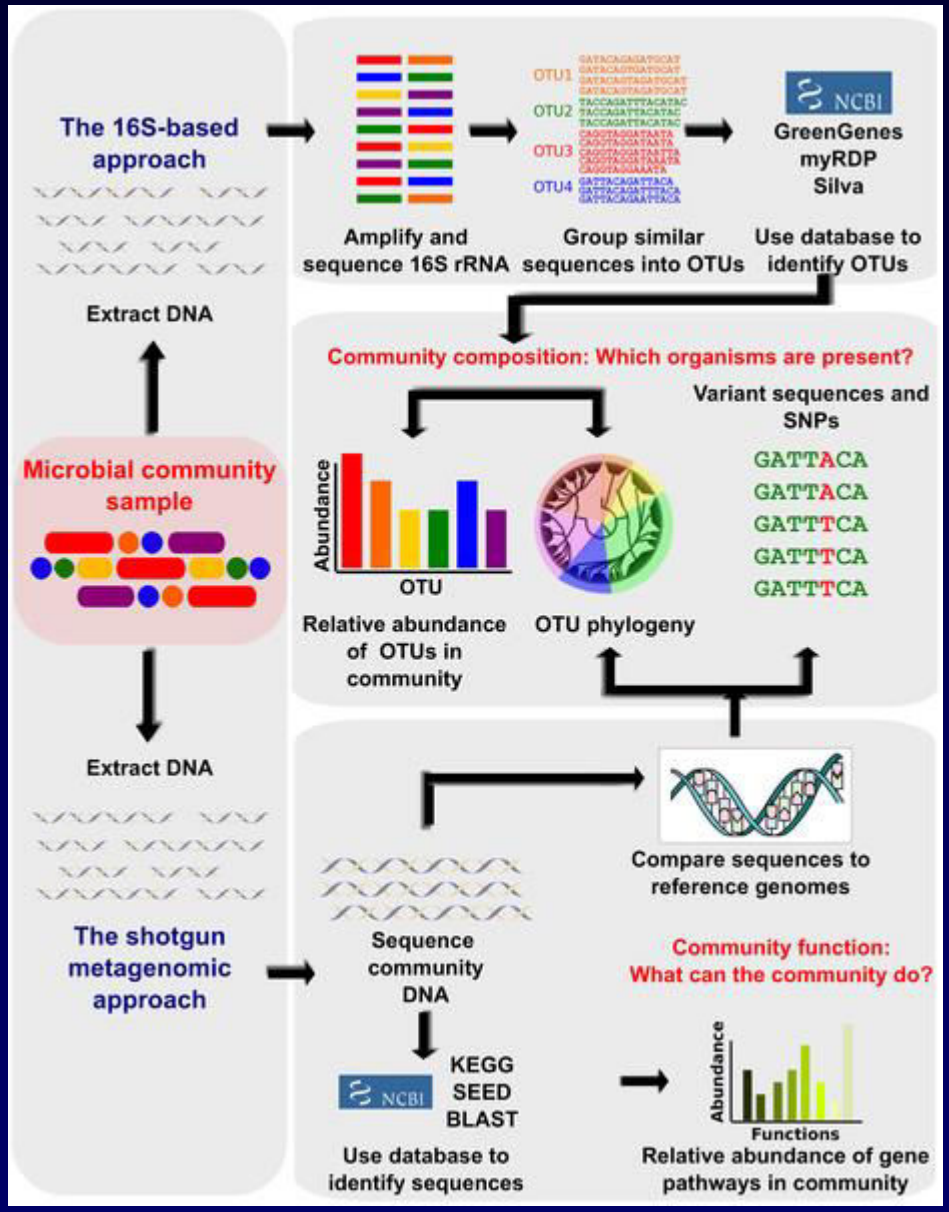
Specific probe for *Vibrio* spp.

Vibrio spp.	AGGCCACAACCTCCAAGTAG Cy3-AGGCCACAACCTCCAAGTAG	Tm (°C) 59.4	Eilers et al. (2000b)

Phylogenetic Analysis



Metagenomics Approach

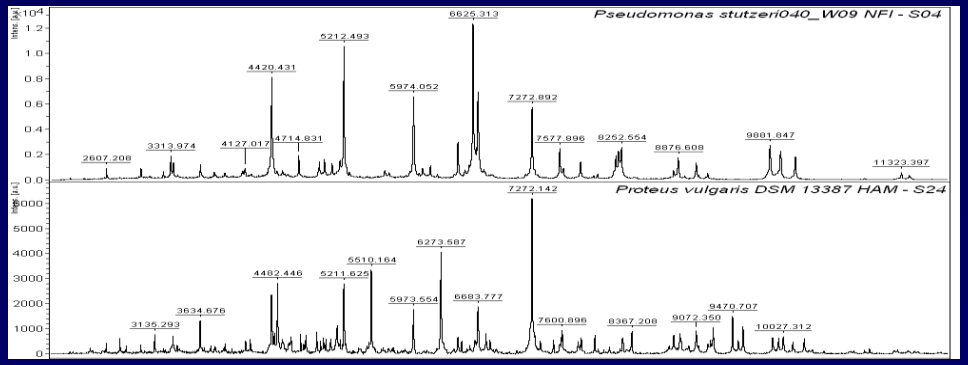


Microorganism Identification and Classification Based on MALDI-TOF MS Fingerprinting with MALDI Biotyper

Fig. 1: General workflow for microorganism identification/classification

MALDI-TOF MS

Spotting of bacterial proteins on MALDI plate



MALDI-TOF MS spectra

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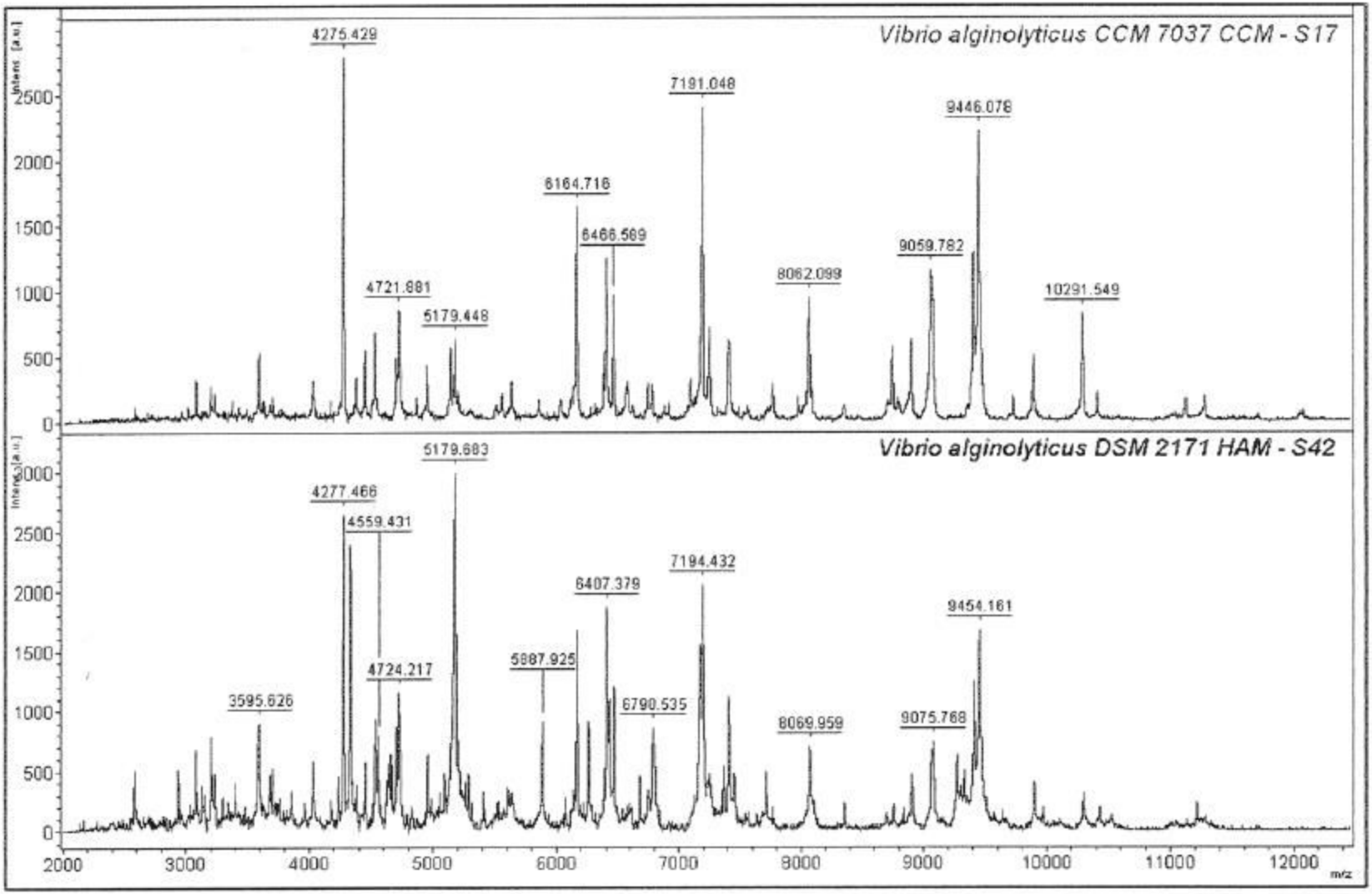

MALDI-TOF mass spectra of *V. alginolyticus* distinguishable by their protein fingerprints

Source: Kaveh E, Vahid A, Matthias U, Khwajah M, AC Anil, Lidita Khandeparker, Burgess JG, Mesbahi E, Plos One (2012)

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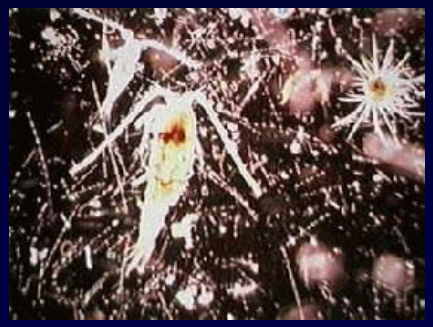
Larvae as hotspots of bacterial diversity – a case study

❖ Marine invertebrate larvae offer extensive body surfaces for attachment of bacteria, their decomposition would contribute to increased bacterial production in the marine environment

❖ Microbial decomposition of larval carcasses serves as an alternate mechanism for nutrient regeneration, elemental cycling and microbial production.

Protocol



Barnacle nauplius, Oithona sp.
& Veliger larva

Sonication

Epibiotic bacteria

Homogenization

Endobiotic bacteria

Bacteria

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Conclusions

✓ Barnacle nauplius and veliger larva harbored ~ 4.4 x 10^5 cells ind^{-1} whereas *Oithona* sp. had 8.8 x 10^5cells ind^{-1}

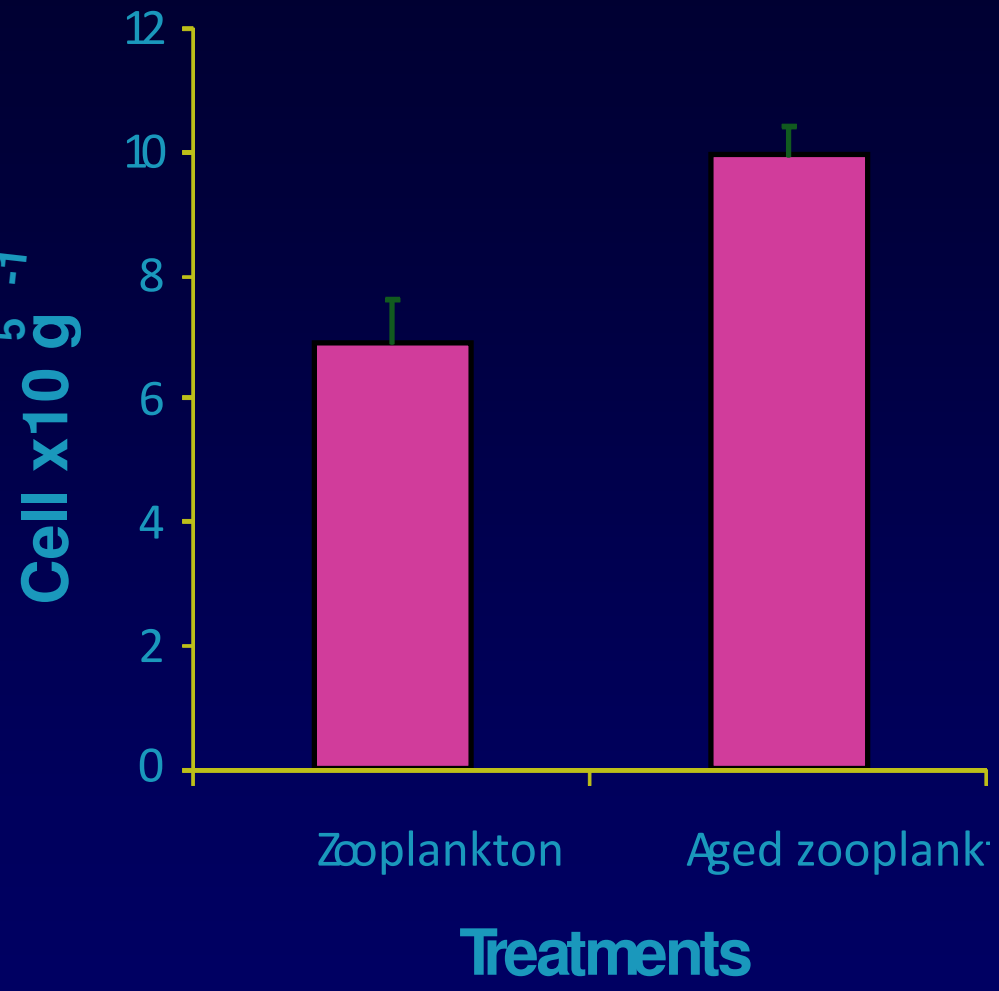
✓ Computation of bacterial contribution based on bio-volume indicated that veliger larva, smallest form of the lot harbored highest numbers of bacteria and barnacle nauplius with largest bio-volume contributed the least

List of epibiotic and endobiotic bacteria specific to different invertebrate forms

Type of organsisms	Epibiotic bacteria	Endobiotic bacteria
Barnacle nauplii	*Aeromonas salmonicida salmonicida*	*Bacillus brevis*
	Aeromonas schubertii	
	Photobacterium angustum	
Copepod	*Aeromonas schubertii*	*Actinobacillus capsulatus*
		Bacillus brevis
Bivalve larva	*Vibrio fischeri*	*Aeromonas schubertii*
	Photobacterium angustum	

Certain degree of form specificity was observed in case of epibiotic and endobiotic bacteria of each of the tested invertebrate forms

Bacterial Abundance on aging



Pathogenic bacteria associated with the zooplankton

Pathogens	Initial (CFU g^{-1})	After pulverization (CFU g^{-1})
Streptococcus feacalis	$2.1 \times 10^2 \pm 4.9 \times 10^1$	$2.5 \times 10^5 \pm 3.8 \times 10^4$
Vibrio cholerae	$3.5 \times 10^3 \pm 1.4 \times 10^3$	$5.4 \times 10^5 \pm 3.1 \times 10^5$
Escherichia coli	$5.0 \times 10^2 \pm 2.8 \times 10^2$	$1.3 \times 10^4 \pm 7.6 \times 10^3$

✓ The numbers of the pathogenic bacteria increased enormously subsequent to zooplankton pulverization

✓ *V. cholerae* which was initially 3.5×10^3 increased to 5.4×10^5 CFU g^{-1} following pulverization of zooplankton. Similar increase was also observed in *E. coli* and *S. faecalis*

Conclusions

✓ After aging of pulverized zooplankton, *Chromobacterium violaceum*, an opportunistic pathogen in animals and humans emerged

✓ Association of pathogenic bacteria in vast numbers with the zooplankton is a cause of concern once they are destroyed or dead

Conclusions

Bacterial numbers were highest in the pre-monsoon season at the Fishing harbour in surface and bottom water and sediments

Lowest bacterial abundance was observed in the sediments of outer port stations in the monsoon and offshore station in the post-monsoon season

Thank you

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