Evaluation of current methods for detecting bacteria in ultrapure water systems - A new look at an old challenge

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Presentation Outline

- Introduction
- Direct filtration (M. McAlister)
  - Epifluorescence microscopy Vs plate counts
  - Molecular based methods (PCR, FISH)
- Endotoxin analysis (M. Gould)
- ASTM standards (E. Gibbs)
- Main conclusions and recommendations
- Interactive questions & answers

Background - Biofilms

- A complex and often highly diversified community of viable and non-viable bacteria, their associated glycocalyx, absorbed organics and entrained particles

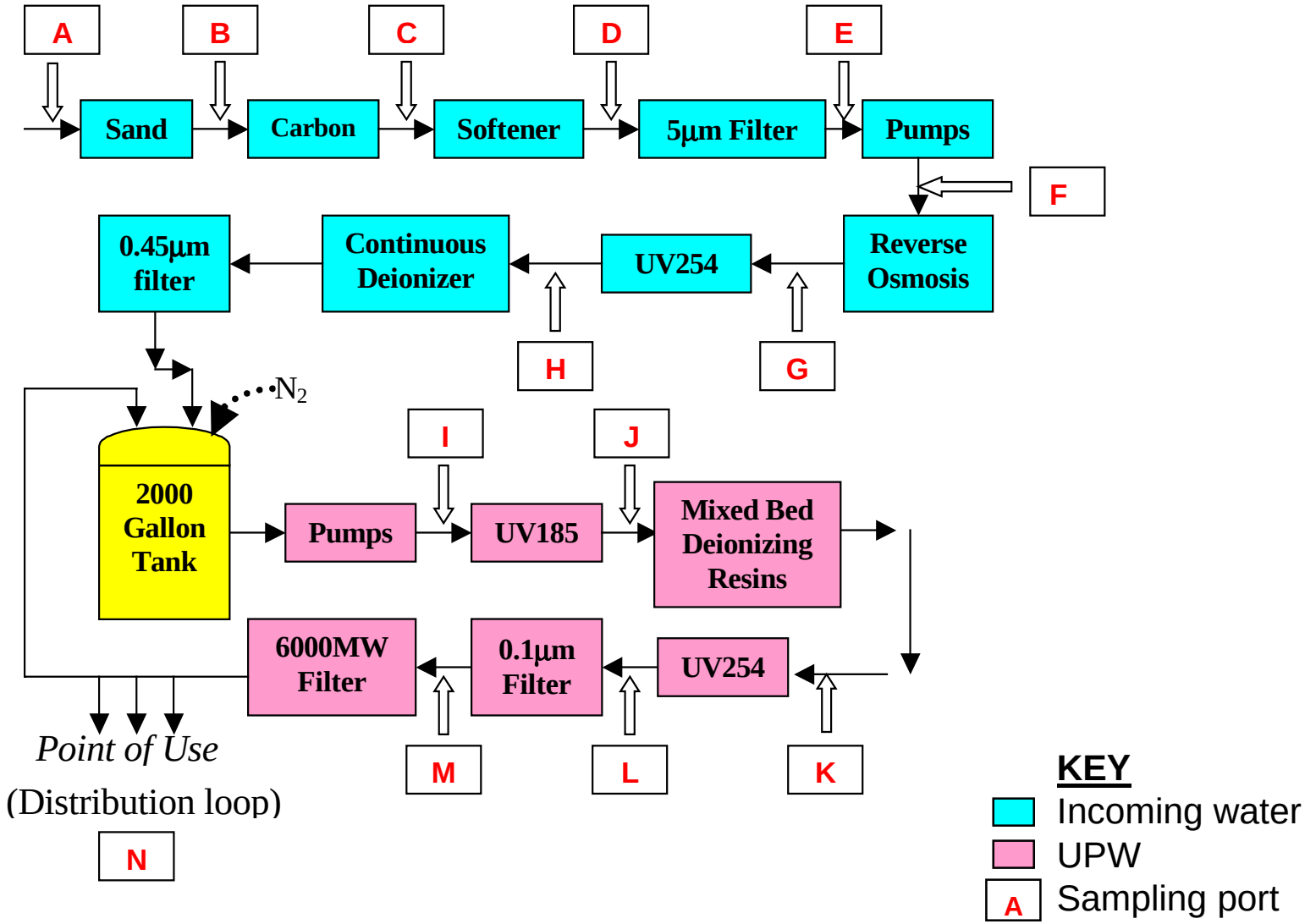
Advantages to Bacteria in Biofilms:

- Protection from shearing
- Constant flow of nutrients
- Increased resistance to antibacterial agents

Background - Oligotrophs

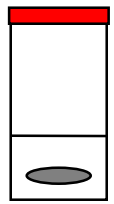
- Cells are often <0.2 μm in size
- May produce extracellular polysaccharides (EPS)
- Ability to scavenge a broad substrate range
- Capable of growth on <1mg carbon/liter
- Often the first bacteria to become established within biofilms

Schematic of UPW System (UA)

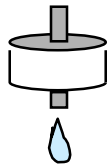


Methodology

Centrifuge

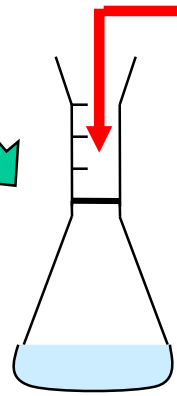


Concentration of bacteria into sterile UPW

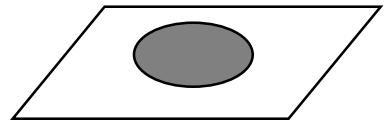


UPW collection

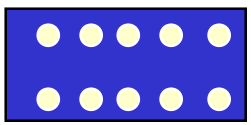
CTC and DAPI applied to membrane



Transfer membrane to filter funnel apparatus

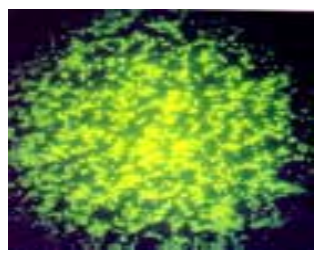


Membrane dried and transferred to microscope slide



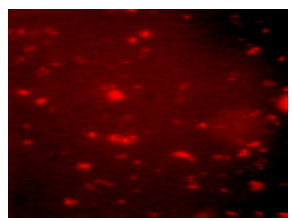
FISH hybridization using primers specific for *Bradyrhizobium* sp. and *P.syzygii*

Examine slide using epifluorescence microscopy



Fluorescein stained cells (+ve hybridization)

Membrane examined using epifluorescence microscopy



No. of total and viable bacterial cells enumerated

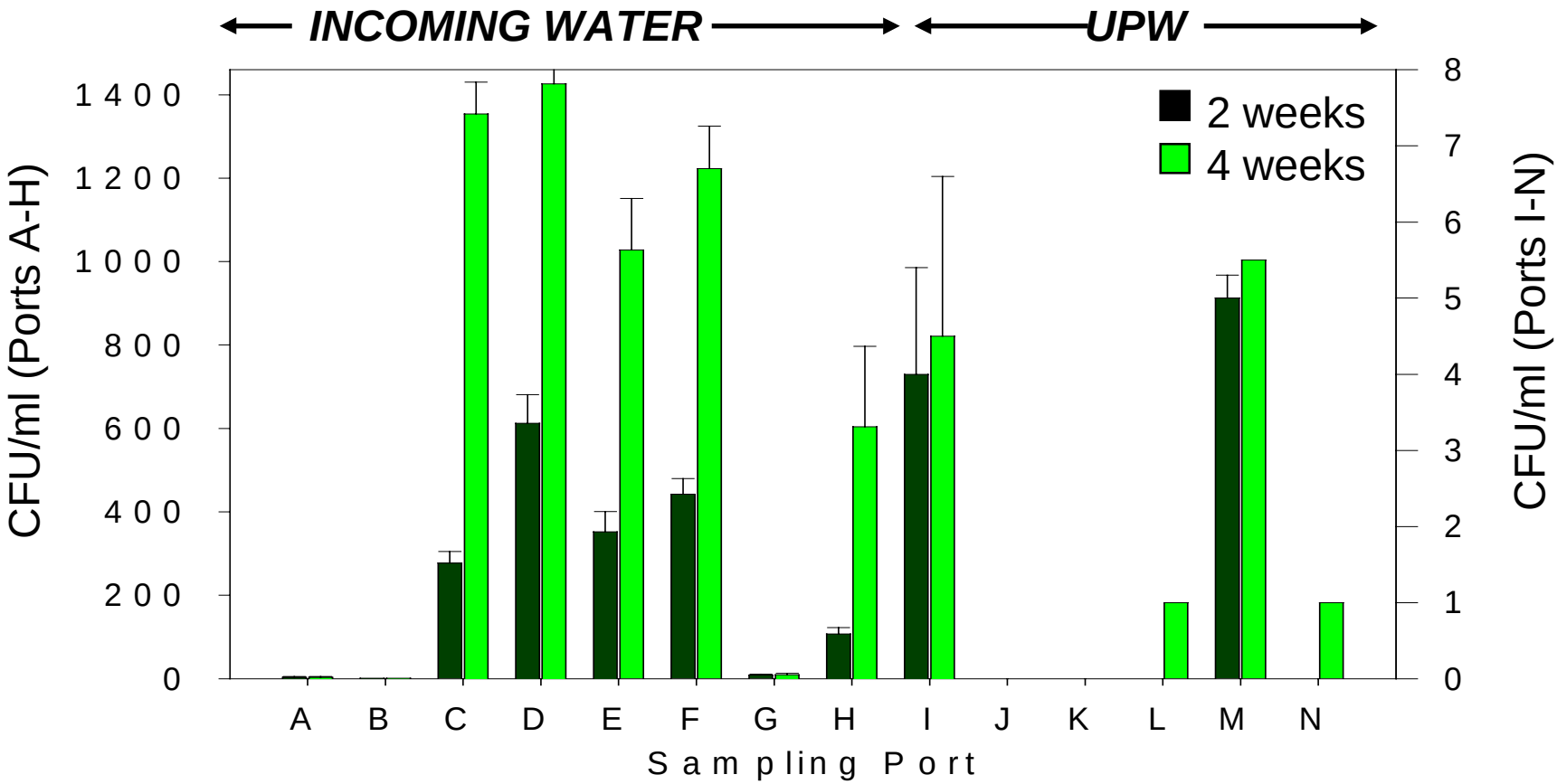
Methodology (Contd)

- Plate Counts *(QUANTITATIVE)*
  - ASTM recommends R2A media and incubation for 48-72 hrs

- Epifluorescence Microscopy *(QUANTITATIVE)*
  - Viable (and potentially viable) count:
    - Cyanotolyl tetrazolium chloride (CTC)
    - Artificial electron acceptors ∴ Reduced within electron transport chain
    - Intracellular formation of red colored formazans
  - Total Count
    - 4',6'-diamidino-2-phenylindole (DAPI)
    - Binds to bacterial DNA
    - Stained cells fluoresce blue under epifluorescence conditions

- FISH/PCR *(QUALITATIVE)*
  - Can be specific or non specific
  - Use probes specific for *Pseudomonas syzygii* and *Bradyrhizobium* sp.

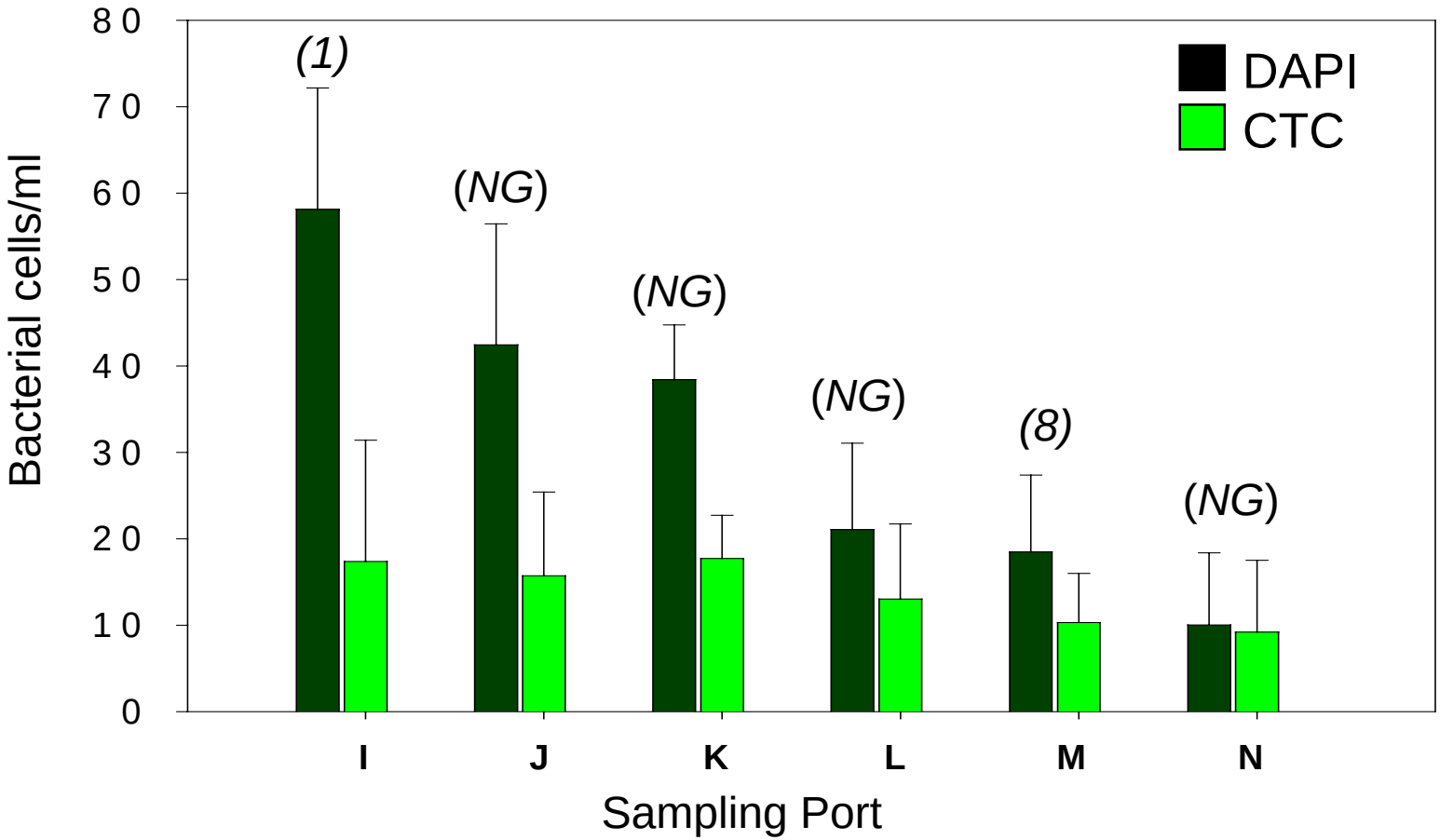
Enumeration of Bacteria in UPW by Plate Counts (R2A) – Effect of Incubation Time



NOTE:

Incubation was at 25°C. All plating was completed in triplicate. Error bars represent the mean standard deviation.

Enumeration of Bacteria in UPW by Epifluorescence Microscopy



NOTE:

Numbers in italics above bars indicate the no. of CFU/ml enumerated by plate counts. *NG;* No Growth. Error bars represent the mean standard deviation.

Plate Counts - Summary

ADVANTAGES:

- Simple methodology
- Minimal equipment required

DISADVANTAGES:

- Highly dependent on growth media and incubation conditions
- May detect only 1-10% of actual bacterial population
  - Stressed bacterial cells
  - Viable but non-culturable (VBNC)
  - Dead cells
  - Unsuitable growth media
- Small sample volume (statistically insignificant)

Epifluorescence - Summary

ADVANTAGES:

- New detection methods significantly faster
  - Plate counts $\rightarrow$ 4 Weeks
  - Direct filtration of water followed by:
    - Epifluorescence microscopy $\rightarrow$ Approximately 4 hours
    - FISH/PCR $\rightarrow$ Within 24 hours
- Increased sensitivity
  - Detection of viable and non-viable bacteria by CTC/DAPI staining
  - Larger sample volume
- Better guarantee of UPW quality

DISADVANTAGES:

- More sophisticated equipment required
- Difficulty establishing “safe” levels of bacteria