Investigations Bootcamp – Addressing Difficult Microbiological Excursions

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Christian Scheuermann
Alex Tan

17/08/2023

Welcome!

Introduction by
Charles River Laboratories

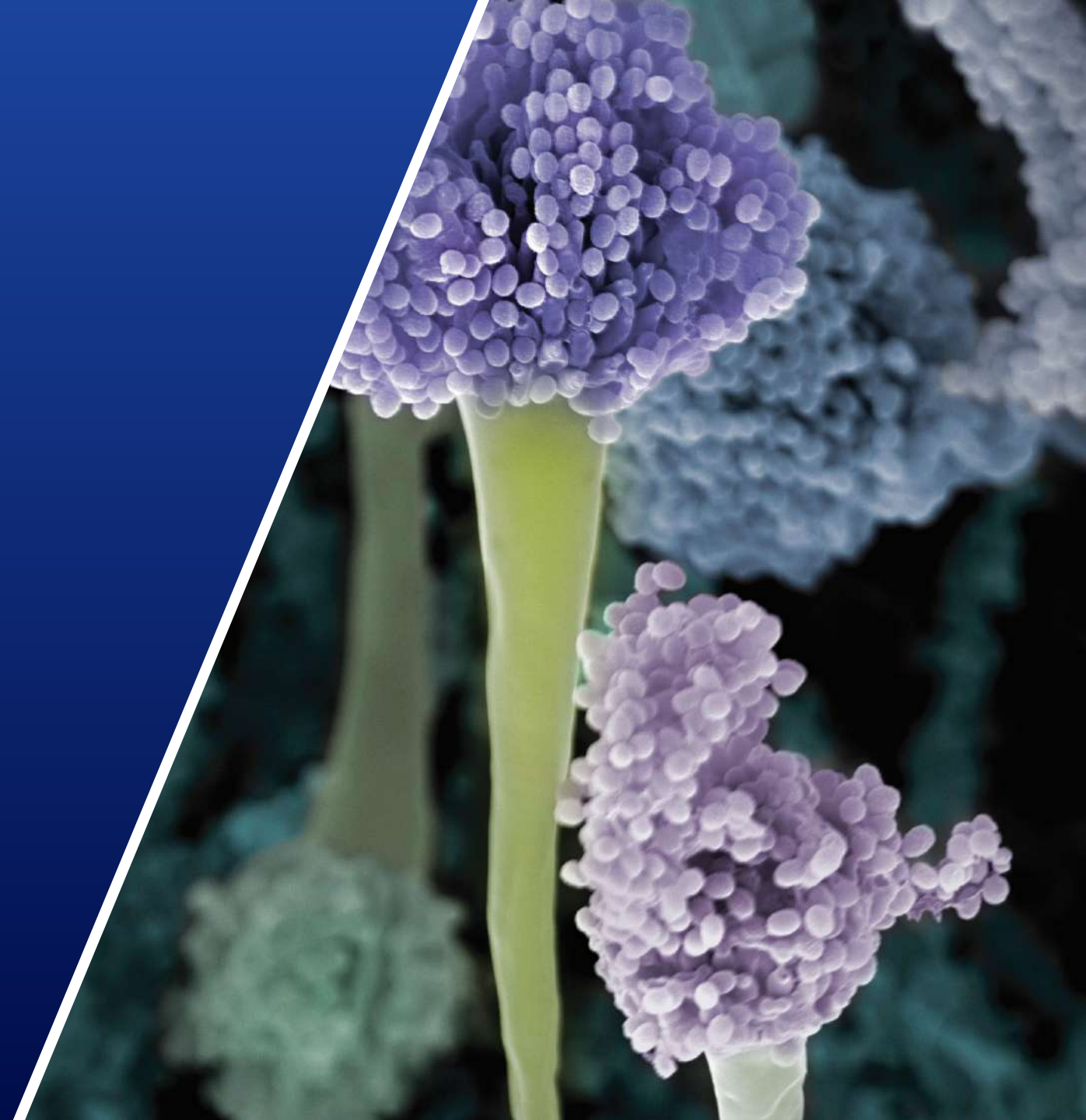
Yong Jian Lee
Technical Services Manager

Today's Agenda

8:30 – 9:00	Registration
9:00 – 9:15	Welcome & Workshop Introduction by Yong Jian
9:15 – 11:15	Contaminants, Causes, Investigation and Case Studies by Ziva Abraham
11:15 – 11:30	Refreshments
11:30 - 12:15	Risk Mitigation with Modern Technologies by Christian Scheuermann
12:15 – 12:45	Difficult to Resolve LAL Investigations by Alex Tan
12:25 – 13:30	Lunch
13:30 – 14:30	Putting it all in practice: Group Breakouts
14:30 - 15:00	Knowledge Sharing Kahoot Game

ASK THE SPEAKERS

PLEASE SCAN QR CODE TO
SUBMIT QUESTIONS



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Speaker Introduction

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Ziva Abraham

CEO of Microrite

Ziva Abraham is the President and Founder of Microrite, Inc., a California based consulting firm providing consulting and training services to pharmaceutical, biotechnology, medical devices, and in vitro diagnostic companies in the areas of quality assurance, quality control, microbiology, and validation. Ziva has over 35 years of academic, research, clinical, and industrial experience in microbiology, and quality assurance. Ziva received her Master's degree in microbiology with a focus on mycology and has conducted research on developing microbial insecticides using entomogenous bacteria and fungi. Her career also includes founding and managing clinical laboratories for Maccabi Medical in Israel. She has trained personnel from various industries in microbiology techniques and methods. She uses her extensive experience to teach why assessing risk of microbial contamination should be in the forefront of any company that has products for human/veterinary use. Her experience in clinical laboratories has provided her with the framework to understand the effects of microbial contamination in products from a patient safety perspective.

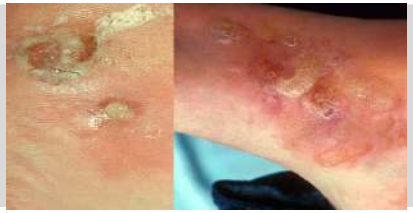
Difficult Investigations

Has mold resurfaced after remediation efforts?

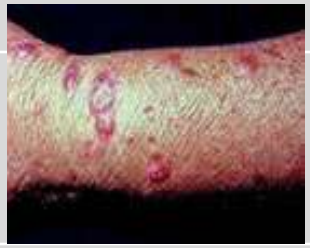
You thought you had it in control!

Clinical Implications of Not Resolving Mold Issues

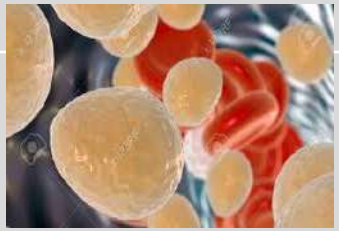
- Cutaneous Mycoses

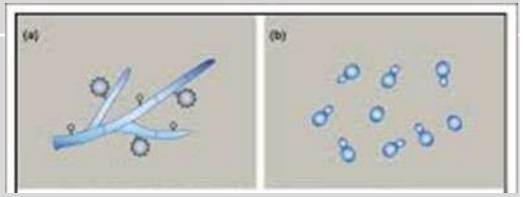


- Opportunistic Mycoses
- Subcutaneous Mycoses

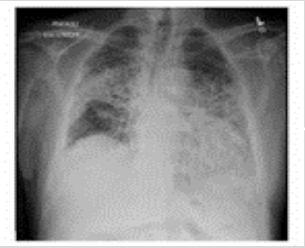


- Keratitis
- Systemic Infections





- Dimorphic Systemic Mycoses



- Pulomonary Mycoses

Never Should Come To This

Teva Pharmaceuticals reportedly recalled 2.5 million vials of drugs and temporarily shut down one of its manufacturing facilities, due to mold contamination and potential sterility problems.

The U.S. supply of saline took a hit as Baxter recalled sodium chloride: nearly 140,000 bags with mold in them.

Never Should Come To This

Bausch & Lomb faced over 600 eye fungus lawsuits

More than 700 lens wearers in the United States and Asia were exposed to a potentially blinding infection known as Fusarium keratitis while using ReNu with MoistureLoc, a new-formula multipurpose solution for cleaning, storing and moistening soft contact lenses.

Sometimes, the damage was irreparable. Seven people in Florida, Maryland, New York, Oregon, Tennessee and West Virginia had to have an eye removed. At least 60 more Americans needed vision-saving corneal transplants.

Never Should Come To This

In 2012, 753 patients in 20 states were diagnosed with a fungal infection after receiving injections of preservative-free methylprednisolone acetate (MPA) manufactured by NECC. Of those 753 patients, the U.S. Centers for Disease Control and Prevention (CDC) reported that 64 patients in nine states died. The government has since identified a total of 793 patients throughout the country harmed by NECC's contaminated MPA. More than 100 patients have now died.

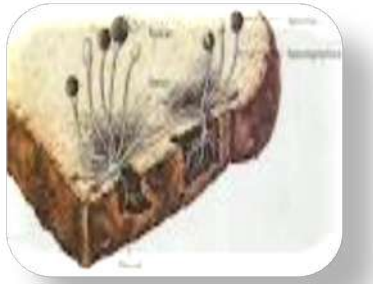
WHO List of Pathogenic Fungi

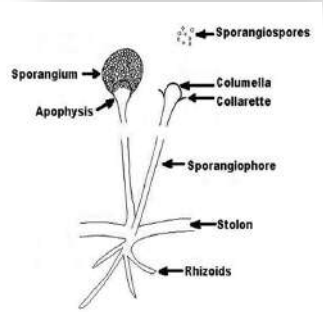
WHO's 2022 list of 19 Fungal species are not the only Fungi that cause various infections

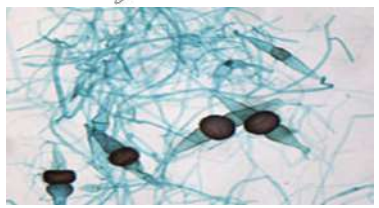
Understanding Fungi

Filamentous Fungi

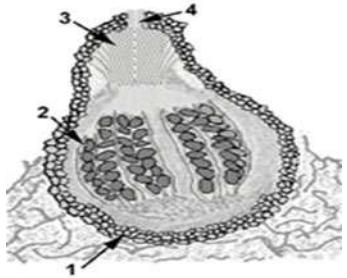
ZYGOMYCOTA

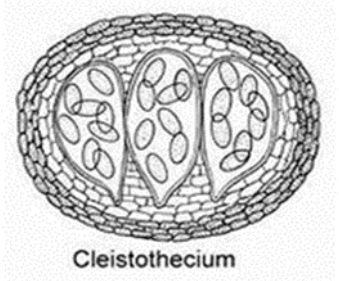






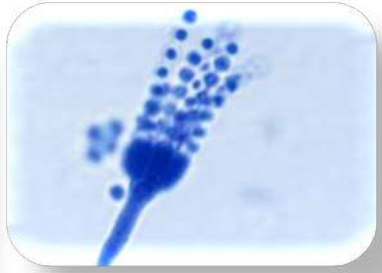
ASCOMYCOTA

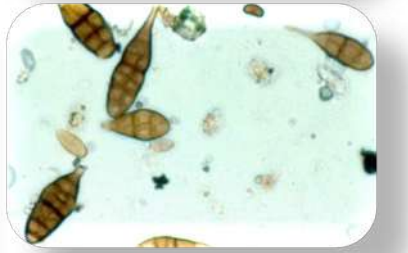




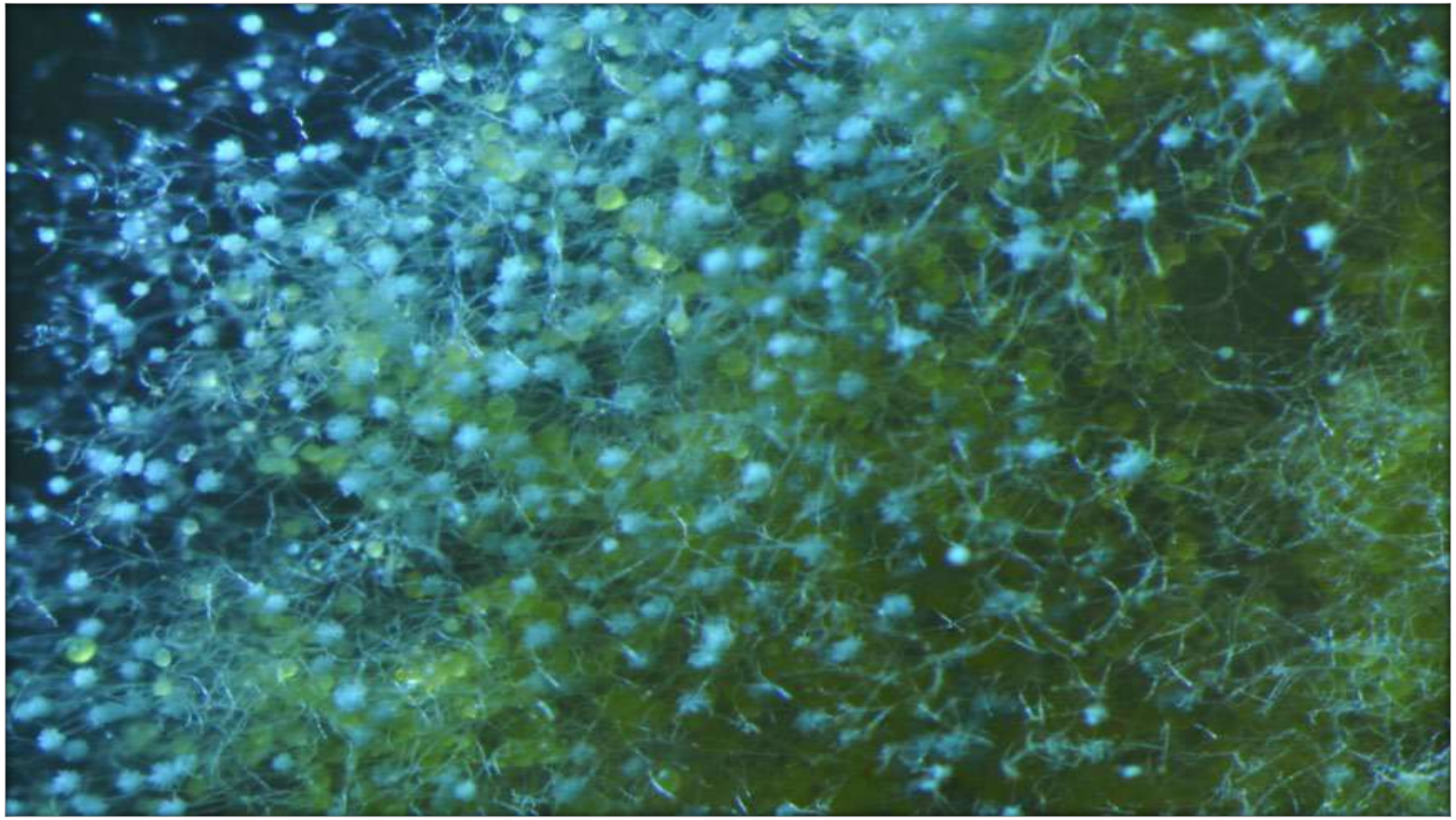
BASIDIOMYCOTA

DEUTEROMYCOTA





Gets Complicated



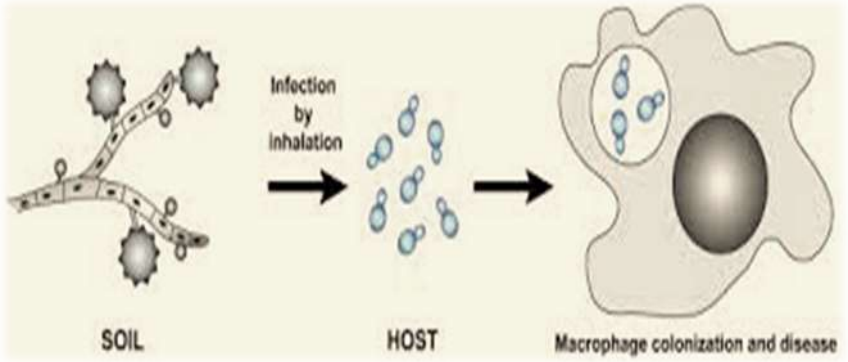
Dimorphism

The fungus occurs in 2 forms

- Mold (filaments) – 25 ° C
- Yeast- 37 ° C

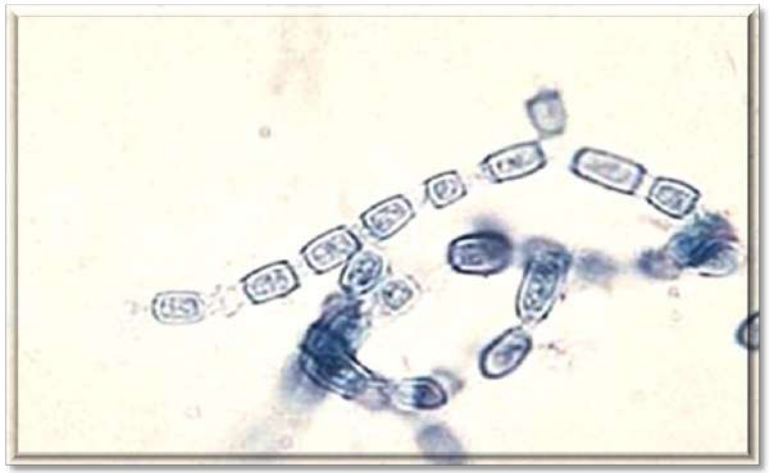
Most fungi causing systemic infections are dimorphic:

- *Histoplasma capsulatum*
- *Blastomyces dermatidis*
- *Paracoccidioides brasiliensis*
- *Coccidioides immitis*
- *Penicillium marneffei*
- *Sporothrix schenkii*

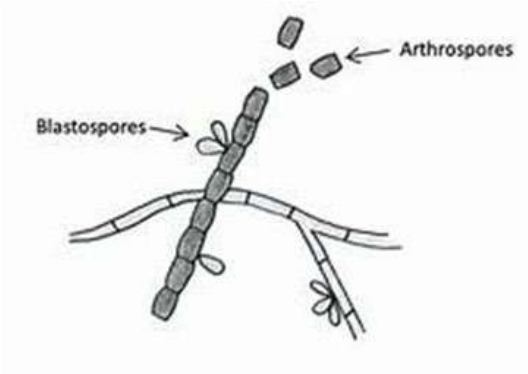


Other Methods of Forming Spores

Chlamydospores



Arthrospores



Arthrospores

Investigation Path

- Know the contaminant
- Analyze the contaminant
- Identify the ingress path
- Map out its transport path
- Verify that it is not proliferating within the cleanroom or product

Understand and Analyze the Contaminant

Knowing the criminal (Contaminant)

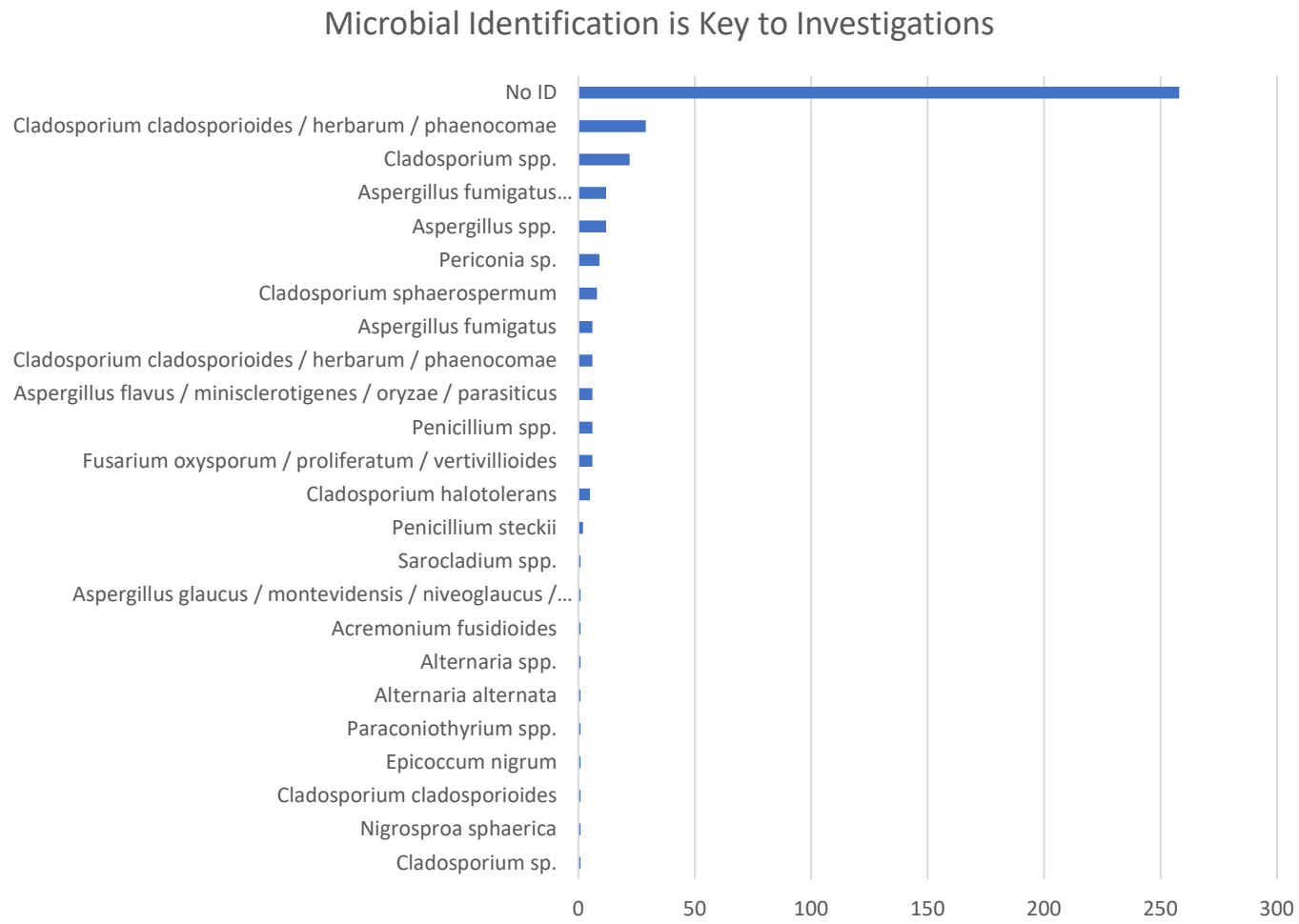
Cannot catch a criminal without a name or first name only!

Identifying the contaminant is the first step.

Cannot presume who the criminal is, valid ID with high level confidence is a must.

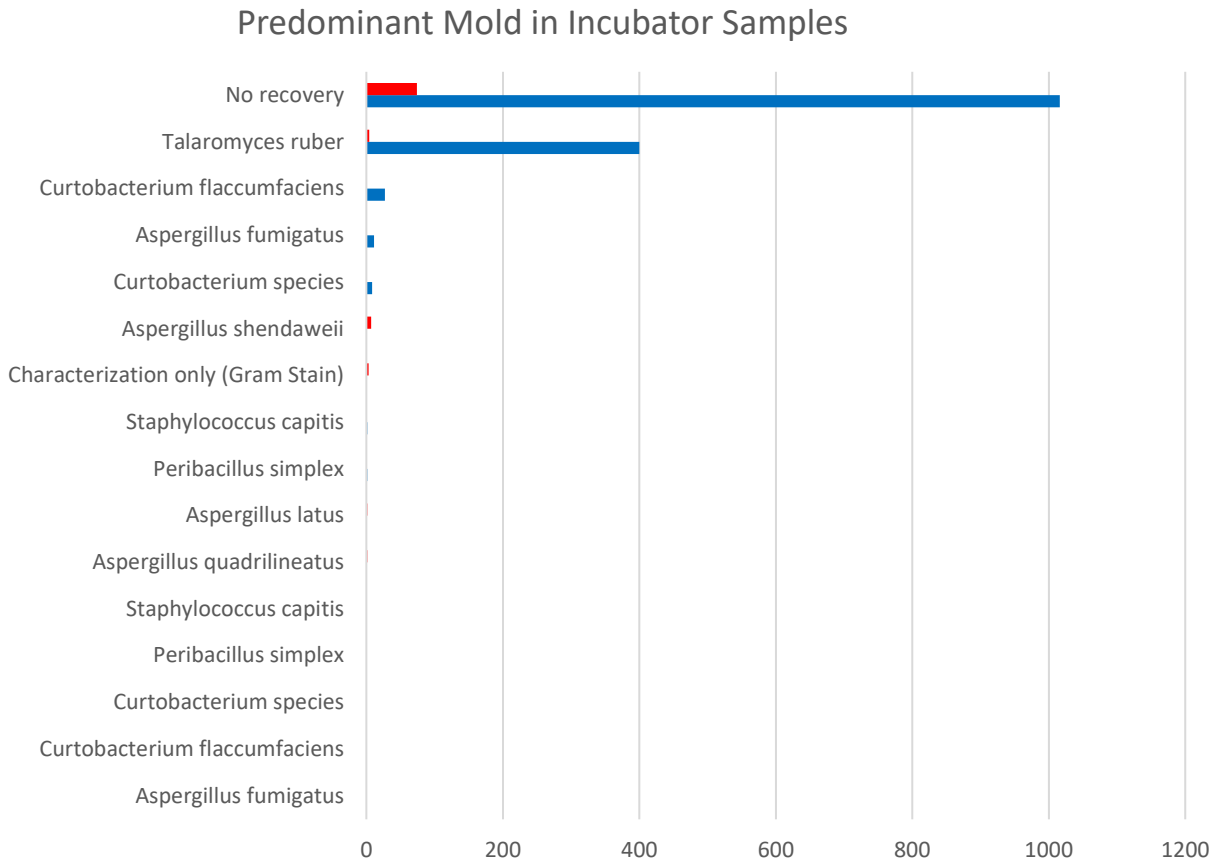
What can happen if the identification is not valid?

How do you investigate without identification?

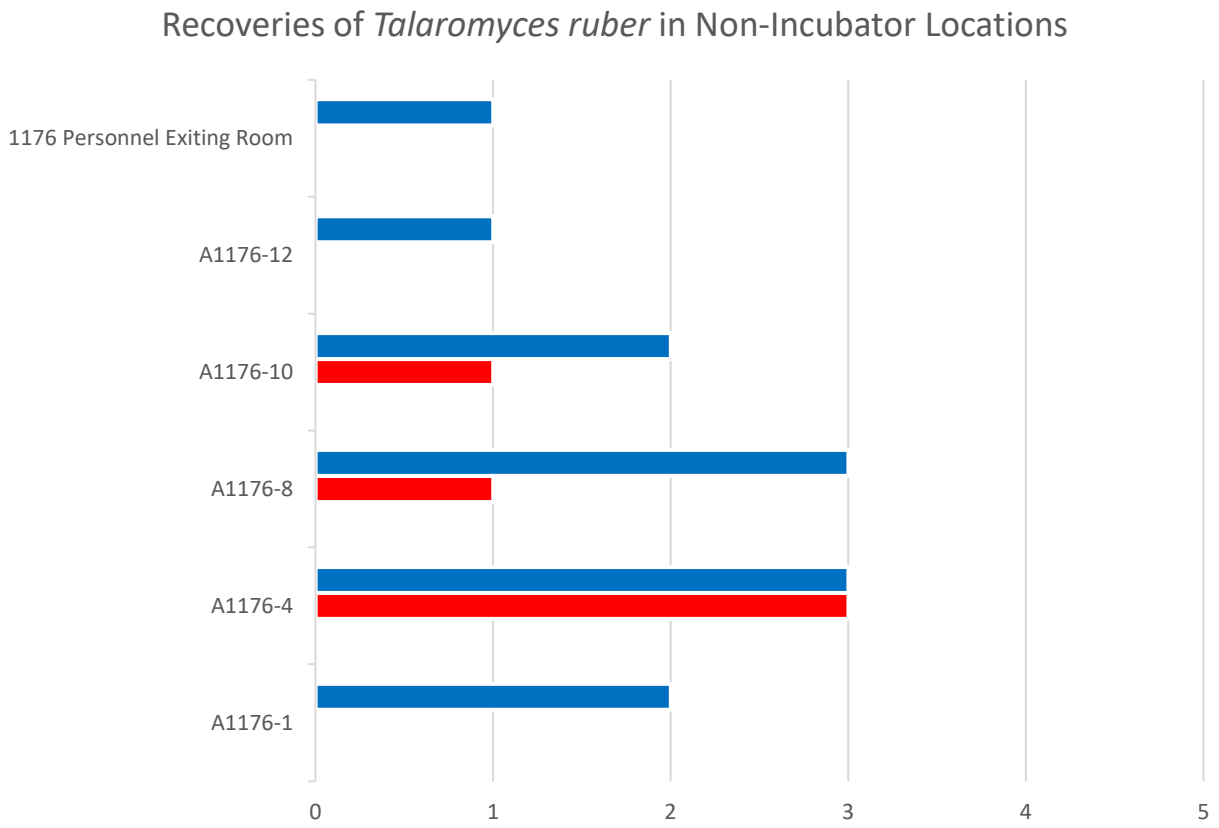


Identification is for investigations, remediation, product quality, stability and patient safety

Know the contaminants



Know the contaminants



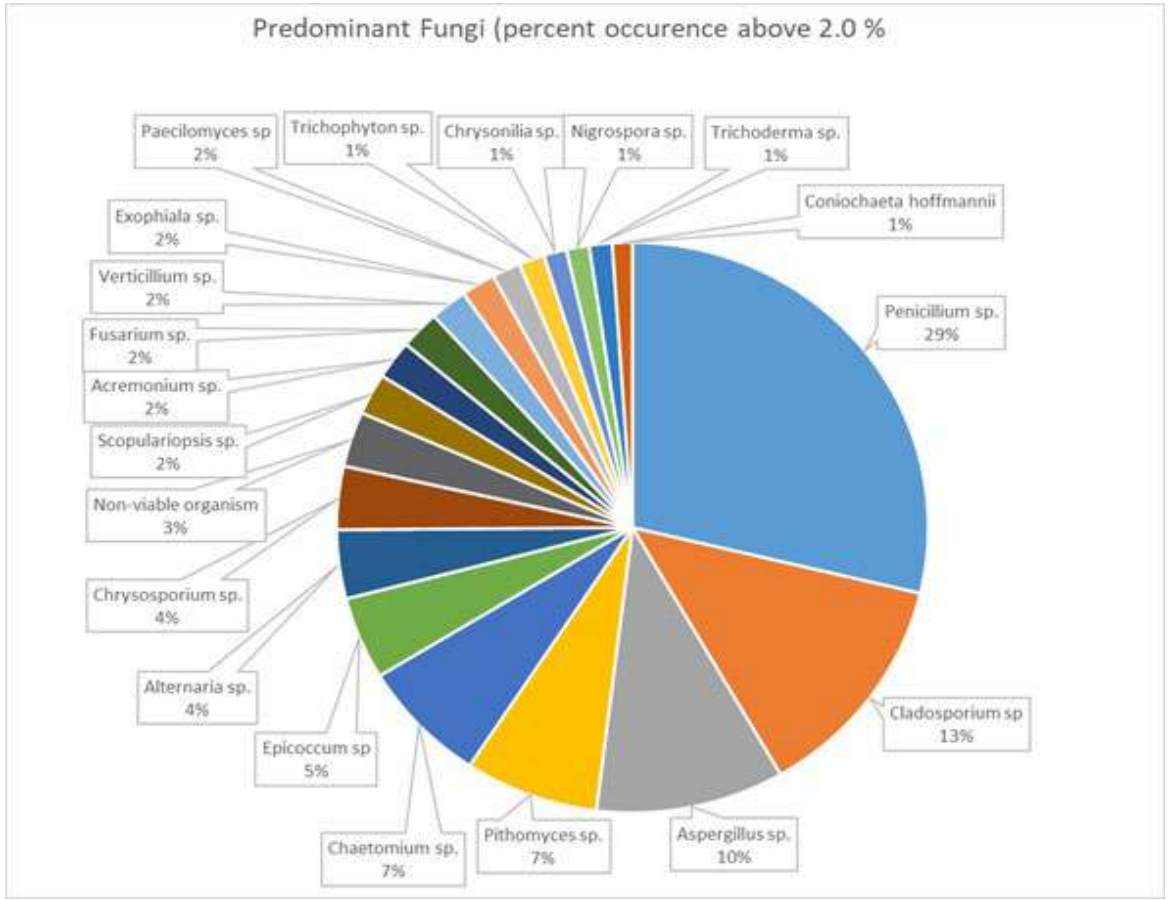
Analyze the Contaminant

How to analyze mold data

Isolate	Division	Anamorph	Teleomorph	Probable source	Growth
Alternaria alternata	Deuteromycota Ascomycota	Alternaria alternata is an anamorph with colored hyphae that belongs to Deuteromycota.	*Clathrospora and Leptosphaeria* are teleomorphs of *Alternaria alternata*	Plant debris, soil, cellulose, and degrading material	Moderately fast growing
Penicillium brevicompactum	Deuteromycota Ascomycota	*Penicillium brevicompactum* is an anamorph with colorless hyphae that belongs to Deuteromycota.	*Eupenicillium, Talaromyces, Hamigera, Trichocoma* are the teleomorphs of *Penicillium brevicompactum*	Soil and decaying vegetation	Fast growing
Cladosporium herbarum	Deuteromycota	*Cladosporium herbarum* is the anamorph with colored hyphae belonging to Deuteromycota.	Teleomorph state of *Cladosporium herbarum* is not identified/known	Rotten plant material, soil Grows in cold temperatures	Fast growth
Aspergillus *sydowii*	Deuteromycota	*Aspergillus sydowii is an anamorph* with colorless hyphae belong to Deuteromycota.	*Emericella sydowii* is teleomorph of *Aspergillus* *sydowii*	Plant debris and soil and cellulose Ubiquitous fungus	Mostly proliferous and fast growing
Aspergillus fumigatus	Deuteromycota	*Aspergillus fumigatus* is an anamorph with colorless hyphae belong to Deuteromycota.	*Neosartorya fumigata* is the teleomorph of *Aspergillus fumigatus*	Plant debris and soil and cellulose. It is an ubiquitous fungus.	Mostly proliferous and fast growing

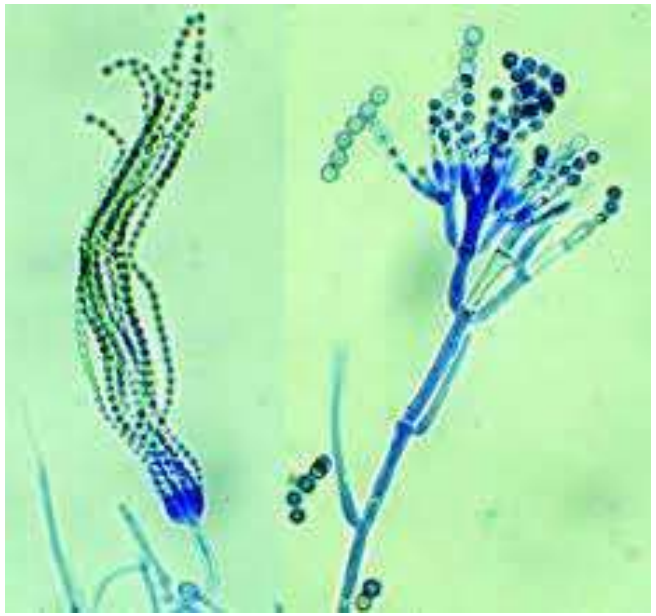
Why the discrepancy in recoveries

Ascomycota and colored Deuteromycete are cellulous; colorless Deutromycota proliferate fast

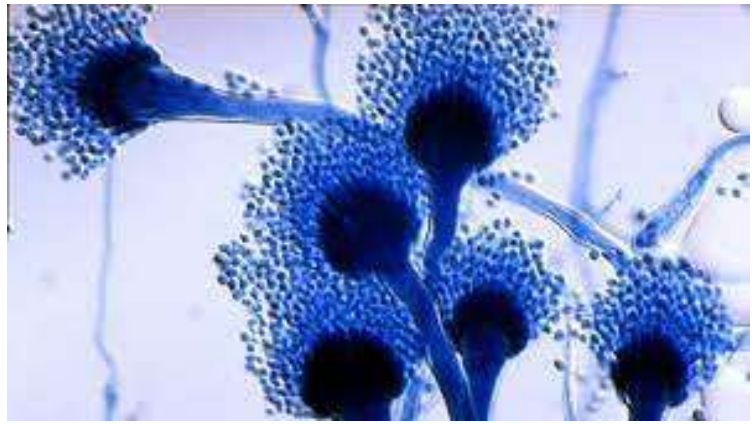


Why the discrepancy in recoveries

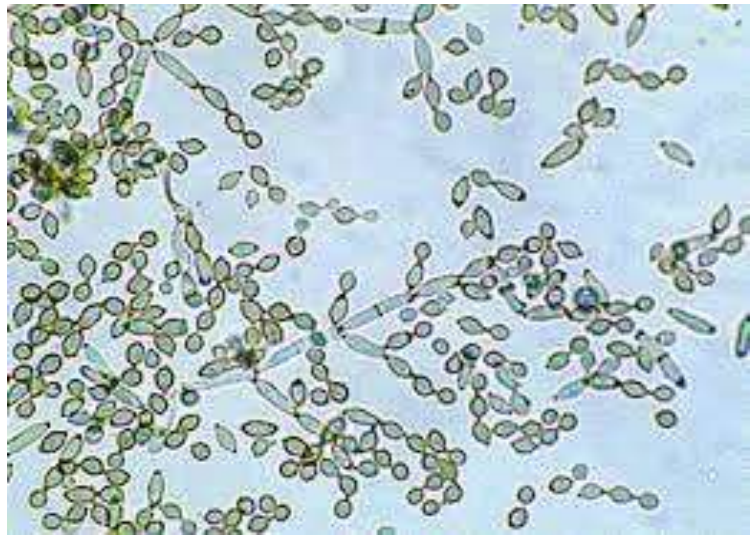
Why *Penicillium*, *Cladosporium* and *Aspergillus* were predominant



Penicillium



Aspergillus



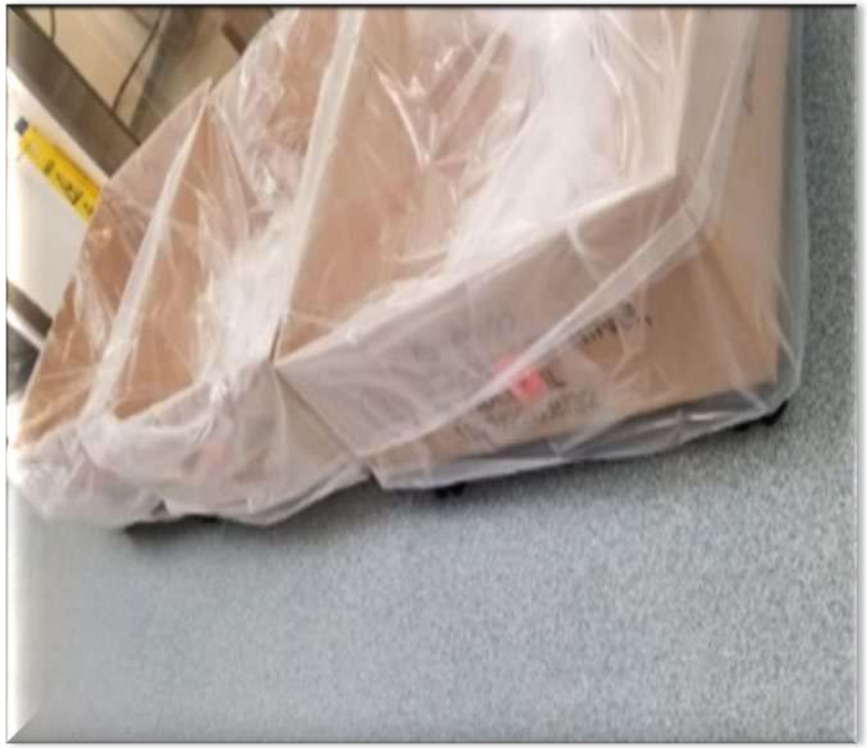
Cladosporium

Ingress

Ingress

Totes can collect mold in warehouse
Tacky mats are not a wheel cleaning solution

Ingress



Source of Cellulous and Other Fungi

Ingress

Coldrooms need to cleaned and maintained

Fungal chandeliers from ceiling tiles in uncontrolled corridor

Ingress

Unclean PPE Bins

Uncleanable areas

Ingress

Fungal chandeliers from ceiling tiles in uncontrolled corridor

Tacky mats removed, but glue not cleaned. Fungi and glue are friends.

Ingress

Unclean Lockers

Compromised ceiling tiles and signs of leaks

Transport

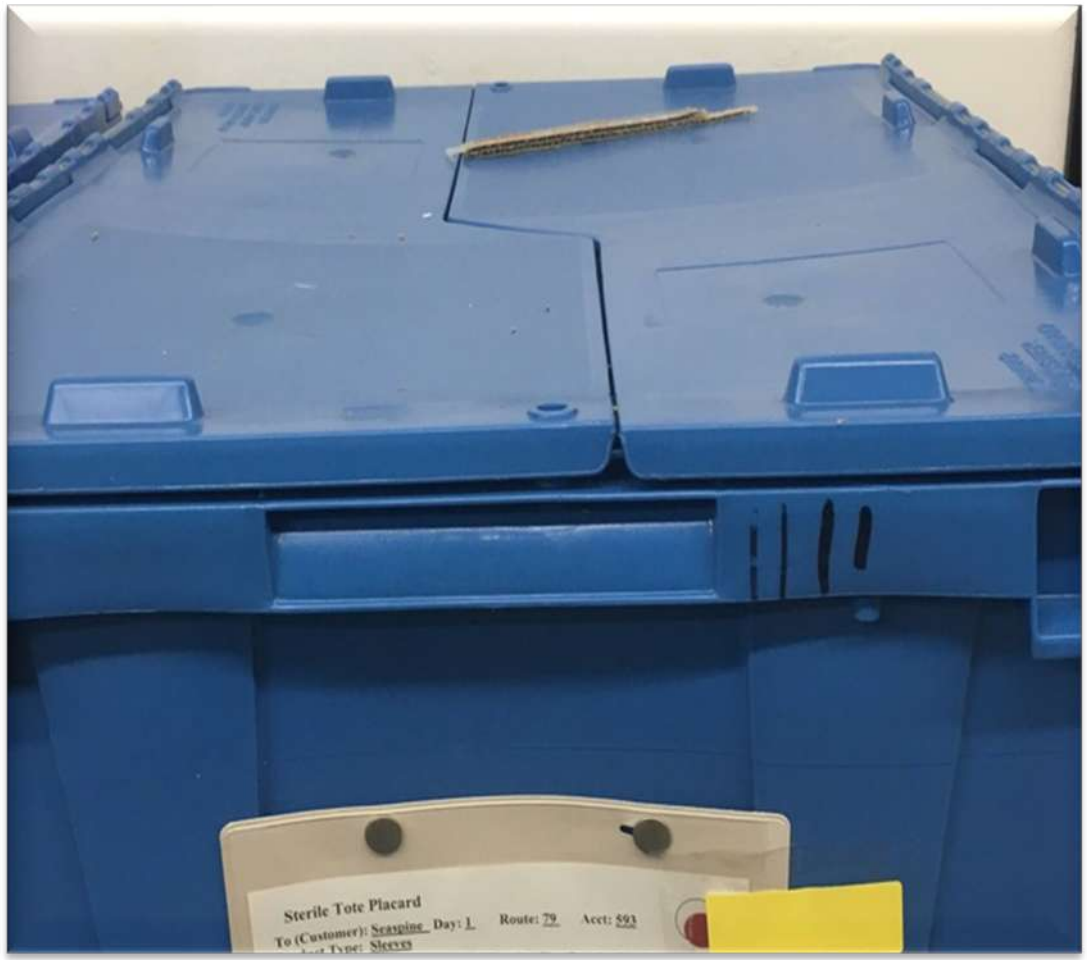
Mold can be transported via:
Wheels
Personnel
On materials
Via airflows

Transport

Tacky mats and Wheels

Transport

Transferred Materials



Transport

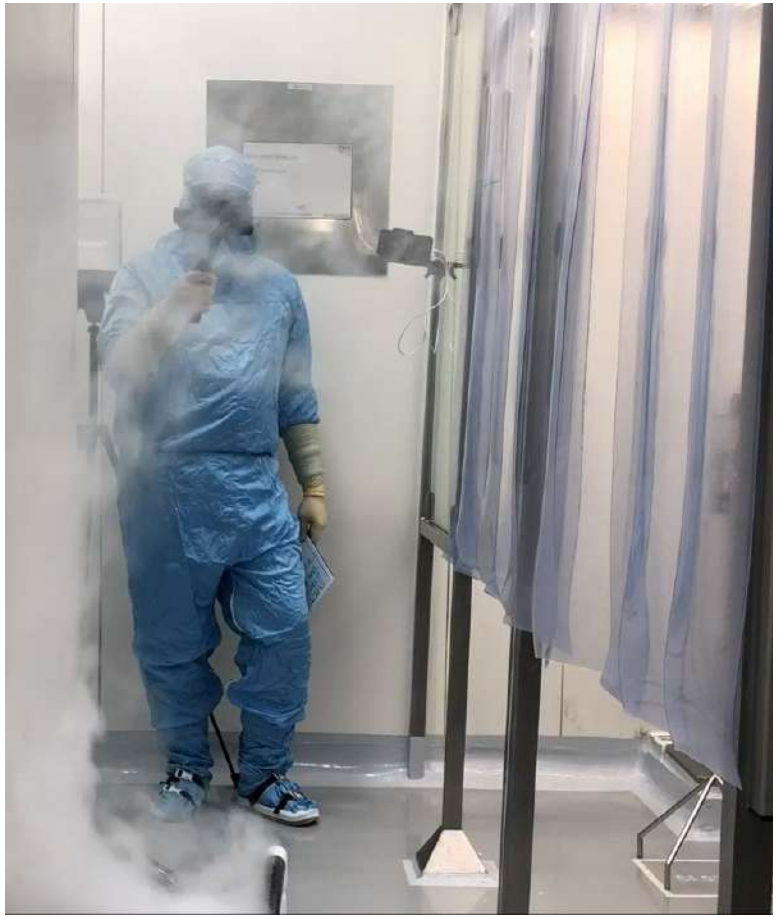
Grade B Gowning Room, Leading into Sterile Processing Room.

Transport

Pass-through from Gowning Room into Cleanroom proximal to BSC.

Transport

Fungi in Cell Therapy Product



Fungi in Cell Therapy Product

Proliferation

Proliferation

Proliferation has happened when:

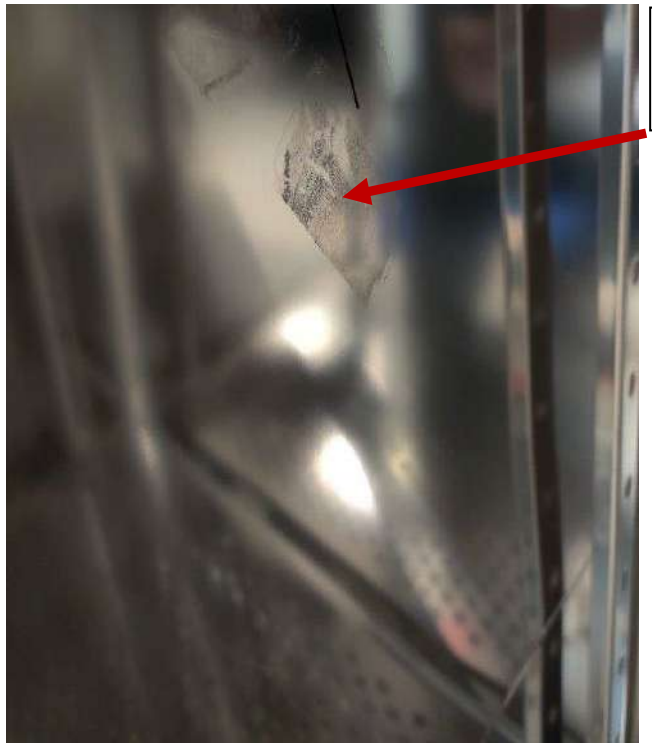
- There are leaks
- There are wet operations
- There is a carbon source for the mold to grow on
- Paper, glue, wet surfaces, organic materials, rags, wet wipes, wipe packages are some ways mold will proliferate within the cleanrooms

Proliferation

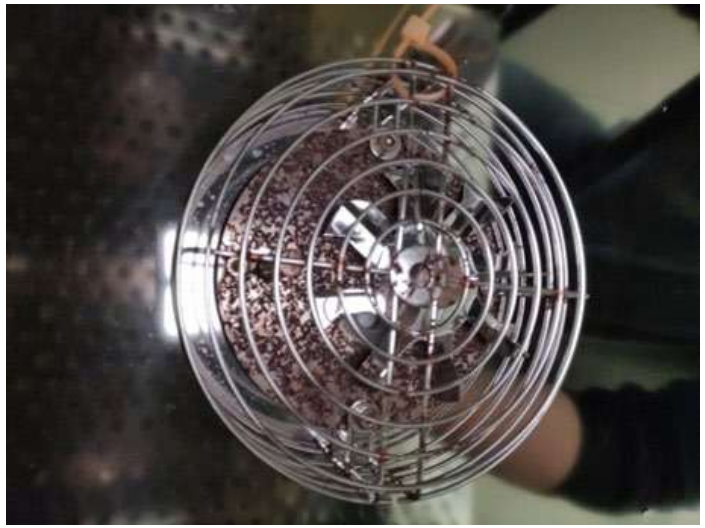
Leak in wall

Mold on labels in coldroom

Proliferation



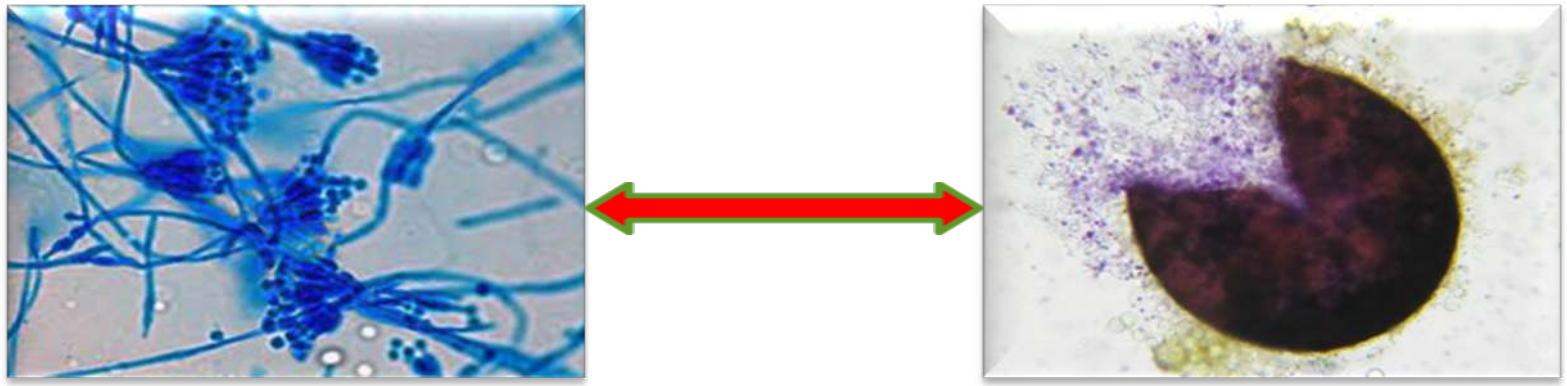
Residual glue is a prominent source of Fungi growth, Fungi uses glue as a carbon source.



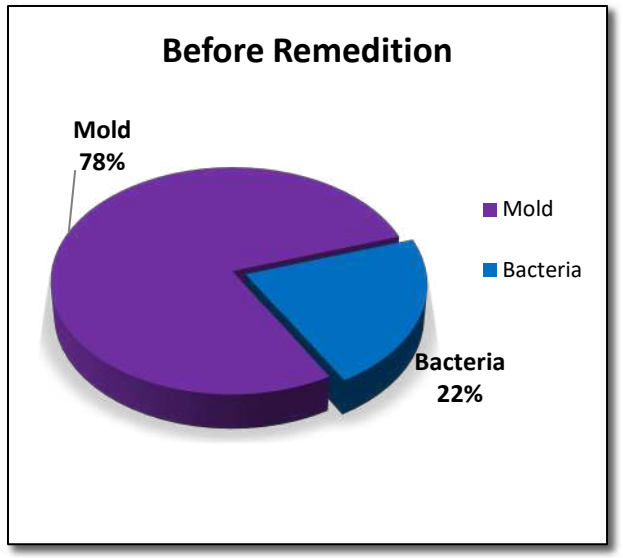
Fan housing seemed rusty. The plastic tie and the entire housing are hard to clean. Once contamination sets in the fan allows dissemination of spores throughout the chamber.

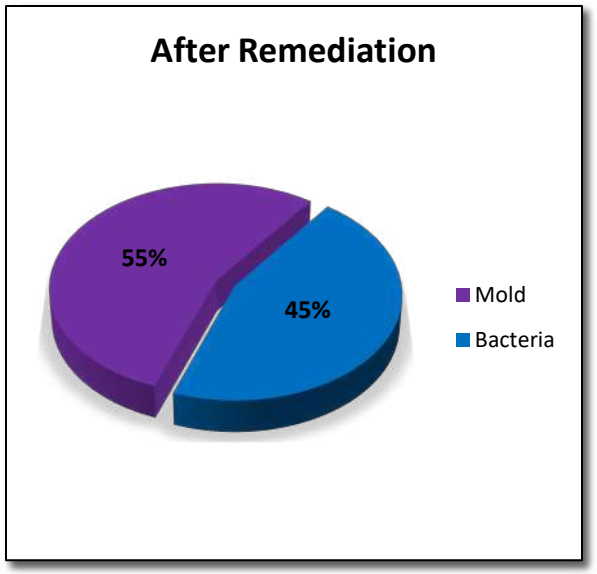
Remediation

Disinfectants are tested for anamorphic stage during label claim testing, the teleomorphic stages are hard to kill



Analyze remediation efficiency





Biofilm

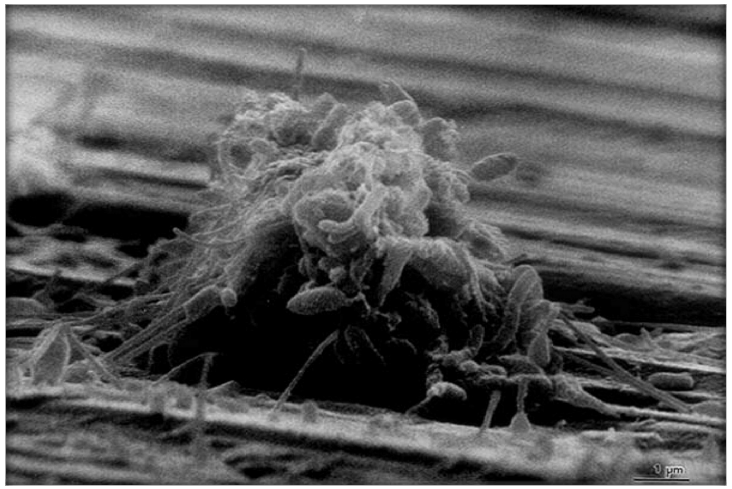
Water Testing Failures are Not Always the Analyst's Fault

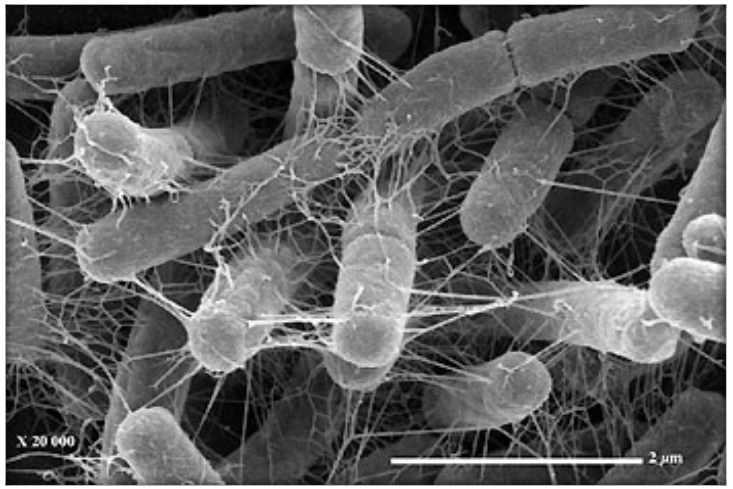
What is a Biofilm?

Three dimensional complex structured community of microbial cells encased in a matrix of extra polymeric substances (EPS)

Most biofilms are attached to a surface in contact with a fluid (sessile cells)

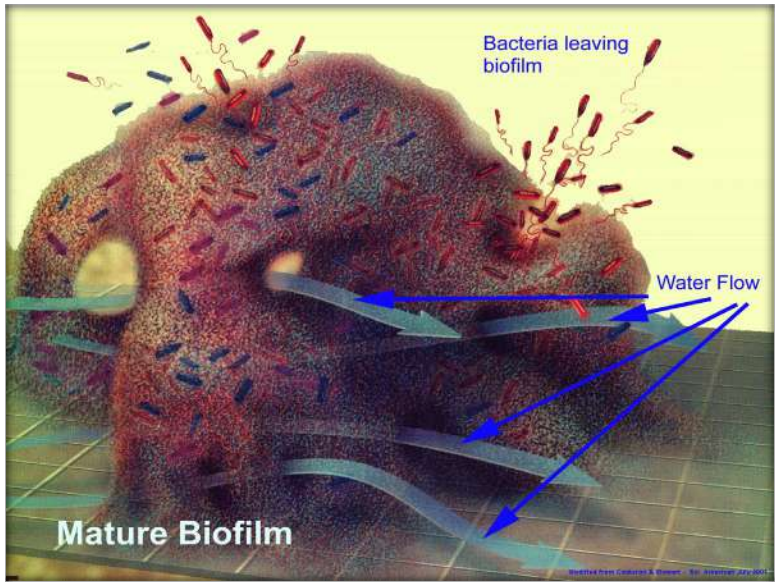
Free-floating aggregates of microbes can also exhibit biofilm characteristics





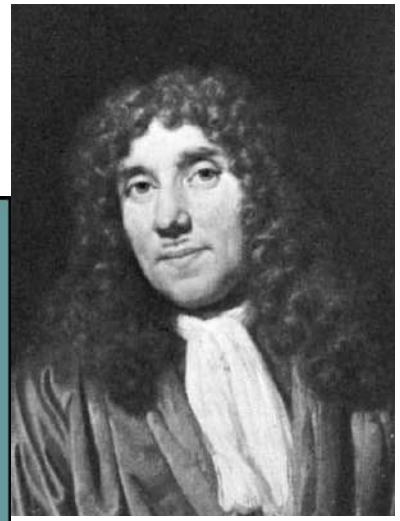
Biofilm Structure

- EPS matrix (~ 85% of biofilm)
  - Carbohydrates, proteins, nucleic acids, lipids, dead cell detritus, entrapped inorganic solids
- Microbial cells (~ 15% of biofilm)
  - Bacteria and fungi
  - Pure and mixed cultures
  - Various metabolic states
- Water channels
  - Nutrients flow in
  - Waste flows out
  - Similar to primitive circulatory system



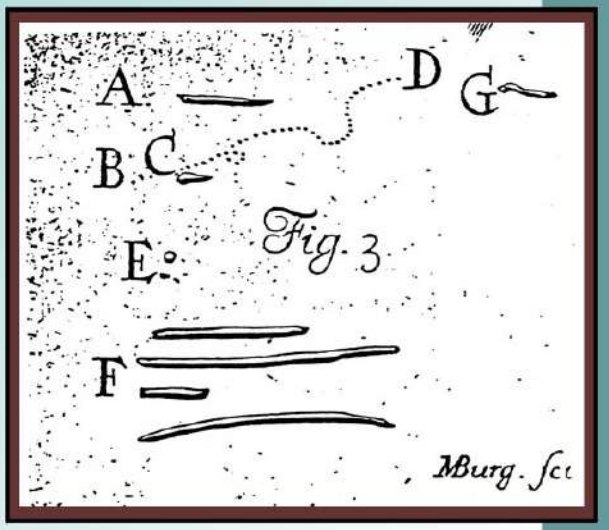
Biofilms - Historical Perspective

First described in the 17th Century



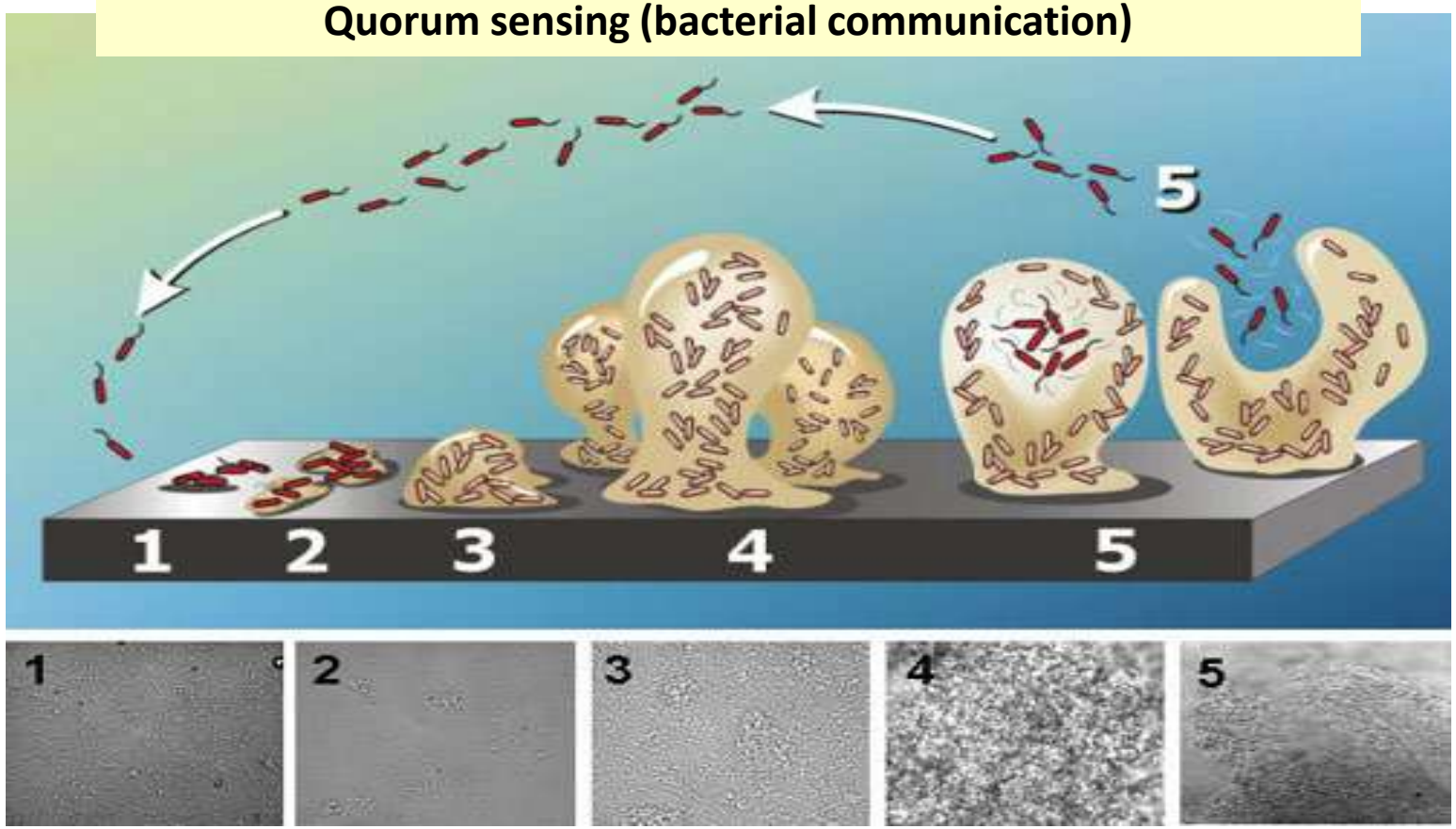
"From whence I conclude, that the Vinegar with which I washt my Teeth, kill'd only those Animals which were on the outside of the scurf, but did not pass thro the whole substance of it."

van Leeuwenhoek, 1684



Stages of Biofilm Formation

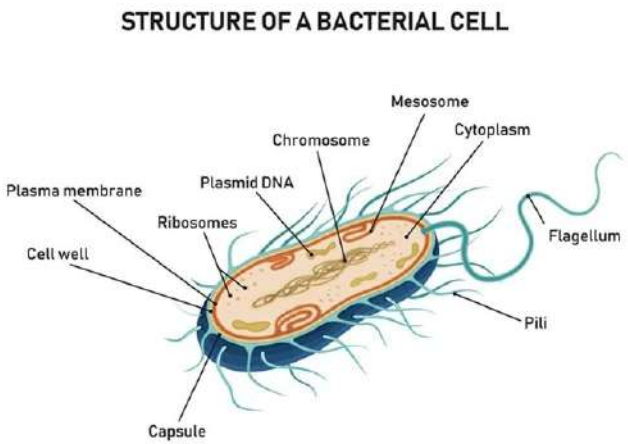
Quorum sensing (bacterial communication)



Bacterial Cell Adhesion

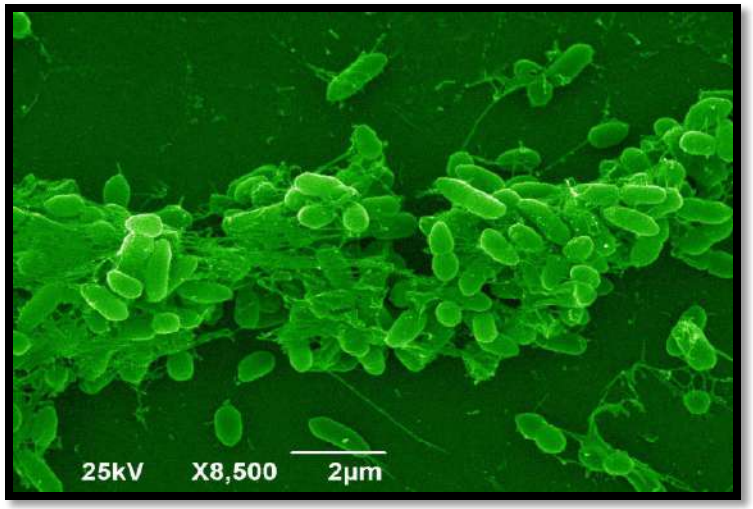
Some factors that influence initial cell attachment

- Conditioning layer/film
  - Proteins and polysaccharides
- Microbial cell properties
  - Cell surface proteins, LPS, pili, flagella
- Biochemical composition of the environment
  - Nutrient concentration in liquid environment
- Hydrodynamics of liquid medium- the study of liquids in motion
- Surface roughness
- Hydrophobicity
- Electrostatic charge

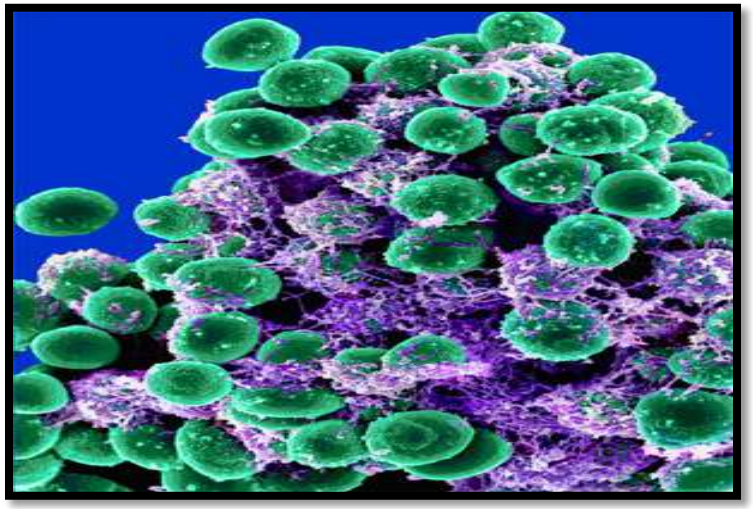


Gram-Negative vs. Gram-Positive Cells

- Both form biofilms!
- Gram-negative bacteria may form biofilms faster
  - Lipopolysaccharide (LPS) is an adhesion factor
  - Many organisms are motile (flagella) and waterborne



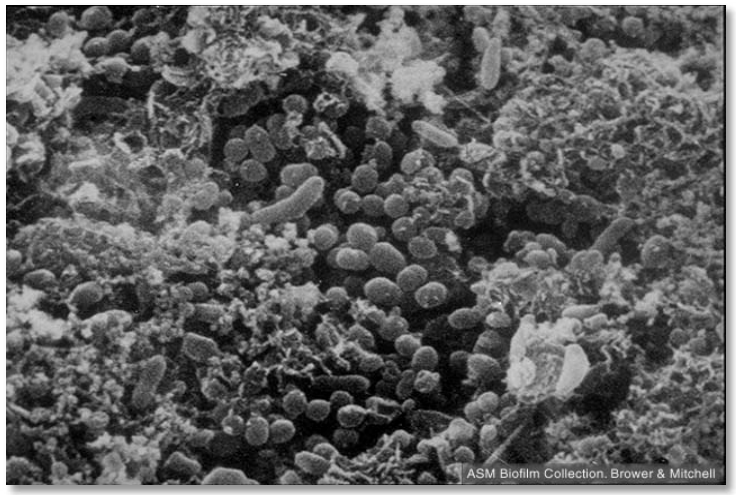
Pseudomonas biofilm



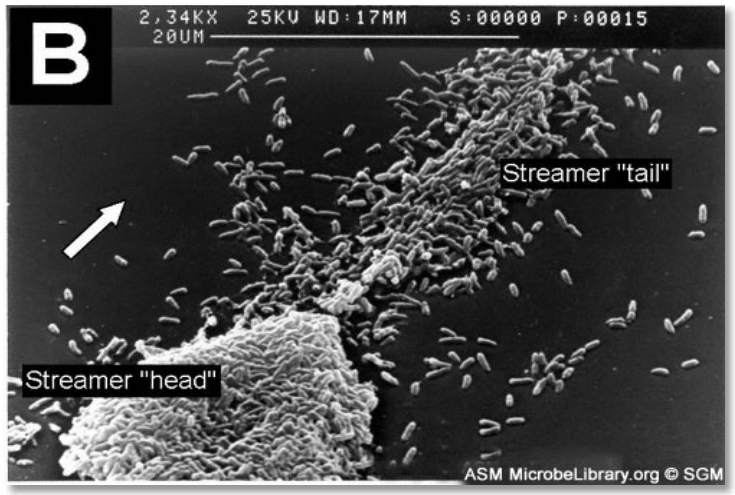
Staphylococcus biofilm

Laminar vs. Turbulent Flow

- Biofilms can form in laminar (low-shear) and turbulent (high-shear) environments
  - Biofilms formed in laminar or stagnant flow are thick, soft, and come off easily.
  - Biofilms formed in turbulent flow are thin, hardy, and form streamers.



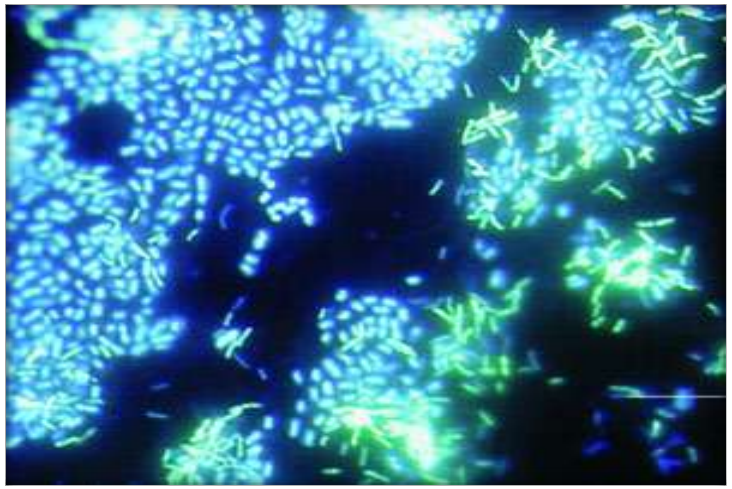
Low-shear biofilm



High-shear biofilm

Bacterial Cell Adhesion

- While factors such as surface hydrophobicity, electrostatic charge, and roughness as well as nutrients and flow dynamics influence rate of *initial* cell attachment, under most circumstances, their impact is reduced over time.



20 µm

14-day old polymicrobic biofilm grown in water on stainless steel coupon using a biofilm reactor

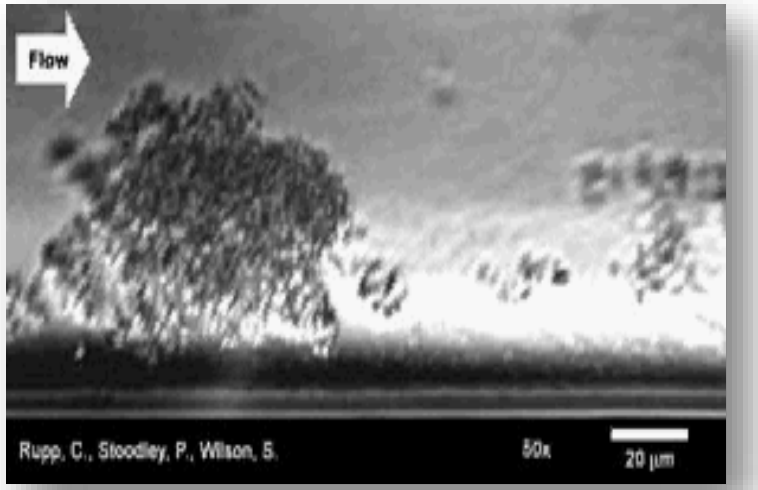
(stain 4,6-diamidino-2-phenylindole (DAPI) for epifluorescence microscopy)

Donlan, R.M. (2002). Biofilms: Microbial Life on Surfaces. *Emerg Infect Dis., 8*(9), 881–890.

Biofilm Detachment/Dispersion

Factors that impact biofilm detachment/dispersion

- Nutrient starvation
- Seeding dispersal
- Surfactants or enzymes
- Quorum sensing
- Fluid shear



Biofilm Resistance

- Biofilm cells are significantly (~ 1000 times) harder to kill than their planktonic counterparts
- Resistance to oxidizing chemicals, non-oxidizing chemicals, and antibiotics
- Example: Gluteraldehyde (50mg/mL) against *P. aeruginosa* biofilm as well as non-tuberculous mycobacteria in devices stored in Gluteraldehyde

Biofilm Contamination

Some common signs of biofilm contamination

- Recurring bioburden of same species, especially gram-negative bacteria
  - Should confirm with identification
- Mixed-culture bioburden
  - Over time, dominant species may change, especially after chemical treatments
- Fluctuating bioburden level
  - Decreased or eliminated with chemical treatment, followed by rapid increase after a few days or weeks
- Sporadic recoveries in water samples
  - Detachment due to hammer effect or maturity and instability

Biofilm Detection

Biofilms are difficult to detect!

- Biofilm cells are less active and more difficult to culture, many can be VBNCs
- Traditional microbiological methods are not suitable for detection or recovery of biofilm cells
- Biofilm cells may be mis-identified using phenotypic microbial identification methods

Biofilm Detection – In-Situ Tests

Analysis of intact biofilms on surfaces

Fouling Cell Technology

- Coupon surfaces mechanically polished to a mirror finish to enable biofilm detection via non-destructive methods

Fouling Cell

Biofilm Detection

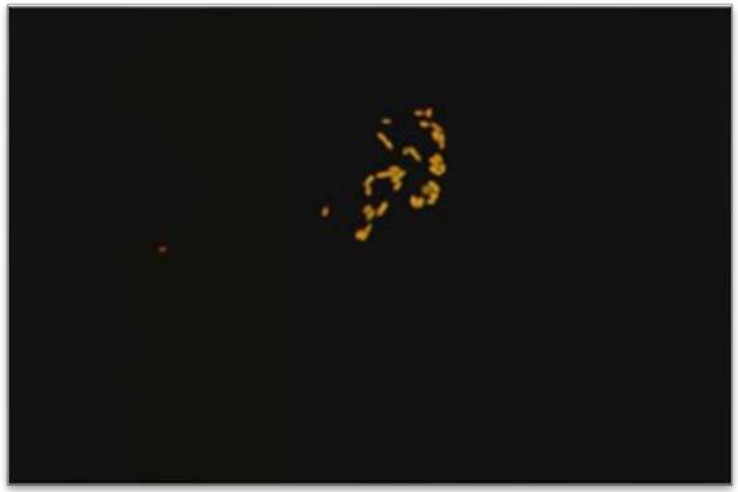
Fouling Cell Technology Biofilm Detection Methods

- Reflection infrared spectroscopy (IR)
- Fourier Transform Infrared (FTIR) spectroscopy
- Atomic Force Microscopy (AFM) to study biofilm morphology
- Ellipsometry to measure biofilm thickness
- Scanning Electron Microscopy (SEM) to study biofilm morphology
- Energy-dispersive x-ray analysis to study elemental composition of biofilm

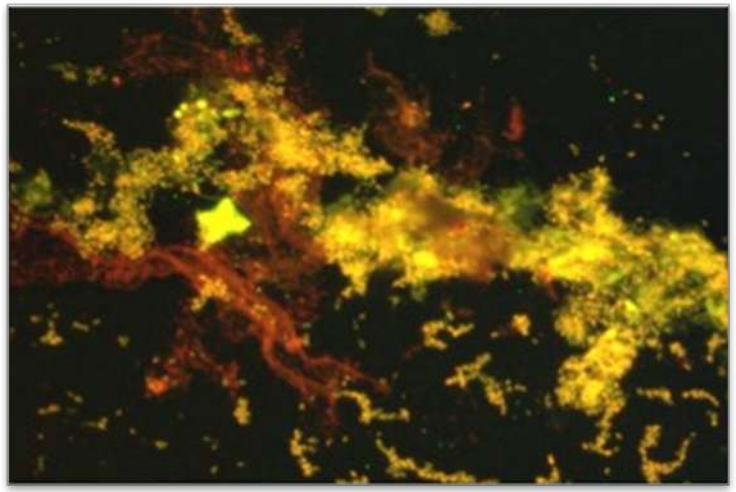
Biofilm Detection – In-Situ Tests

Fouling Cell Technology

- Epifluorescence microscopy of stainless steel fouling cells in high-purity water after 1 and 9 days exposure (100X objective)



1 Day



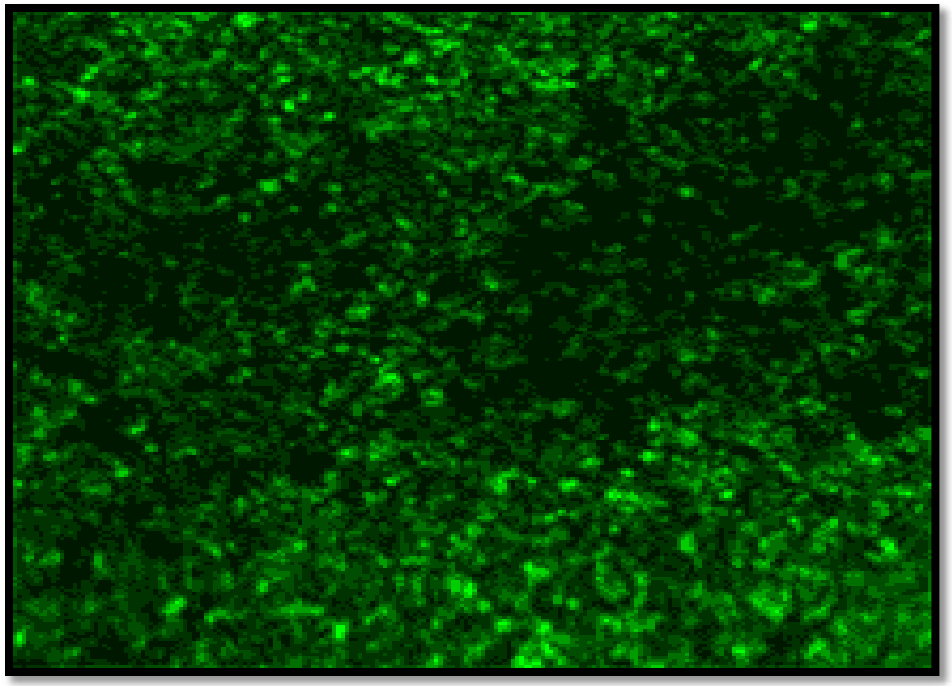
9 days

Biofilm Detection – In-Situ Tests

Confocal Laser Microscopy (CLM)

- *S. pneumoniae* biofilm grown in vitro for 6 days and examined in situ in real time by confocal microscopy.

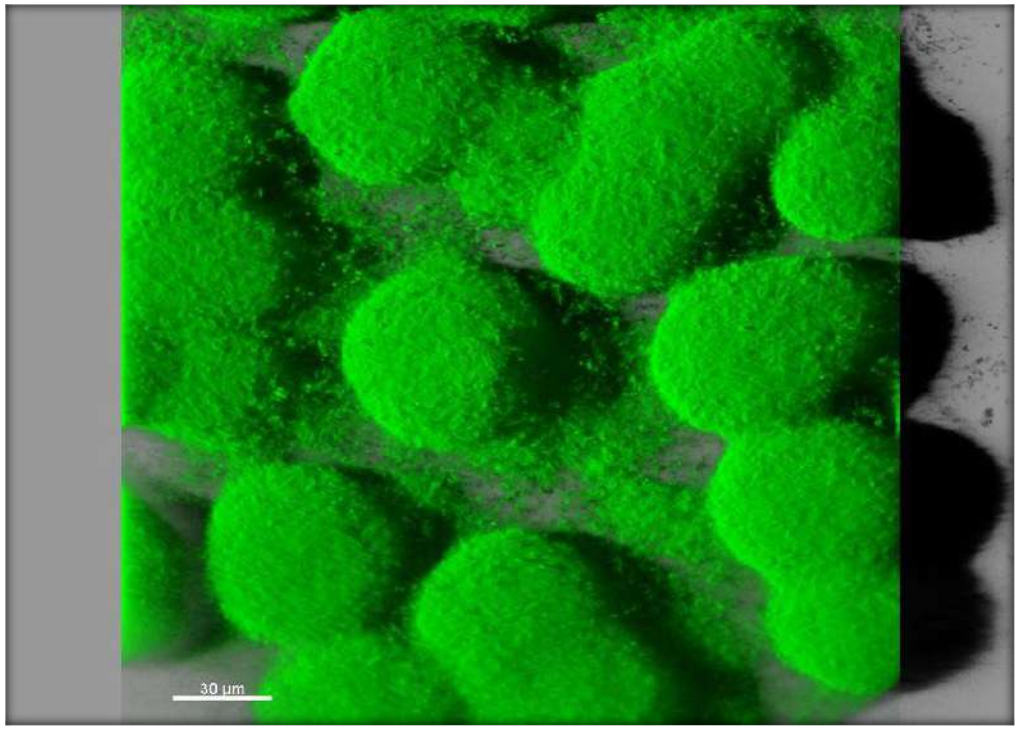
- Detachment of cells and clusters of cells into the surrounding fluid can be seen.



Biofilm Detection – In-Situ Tests

Confocal Scanning Laser Microscopy (CSLM)

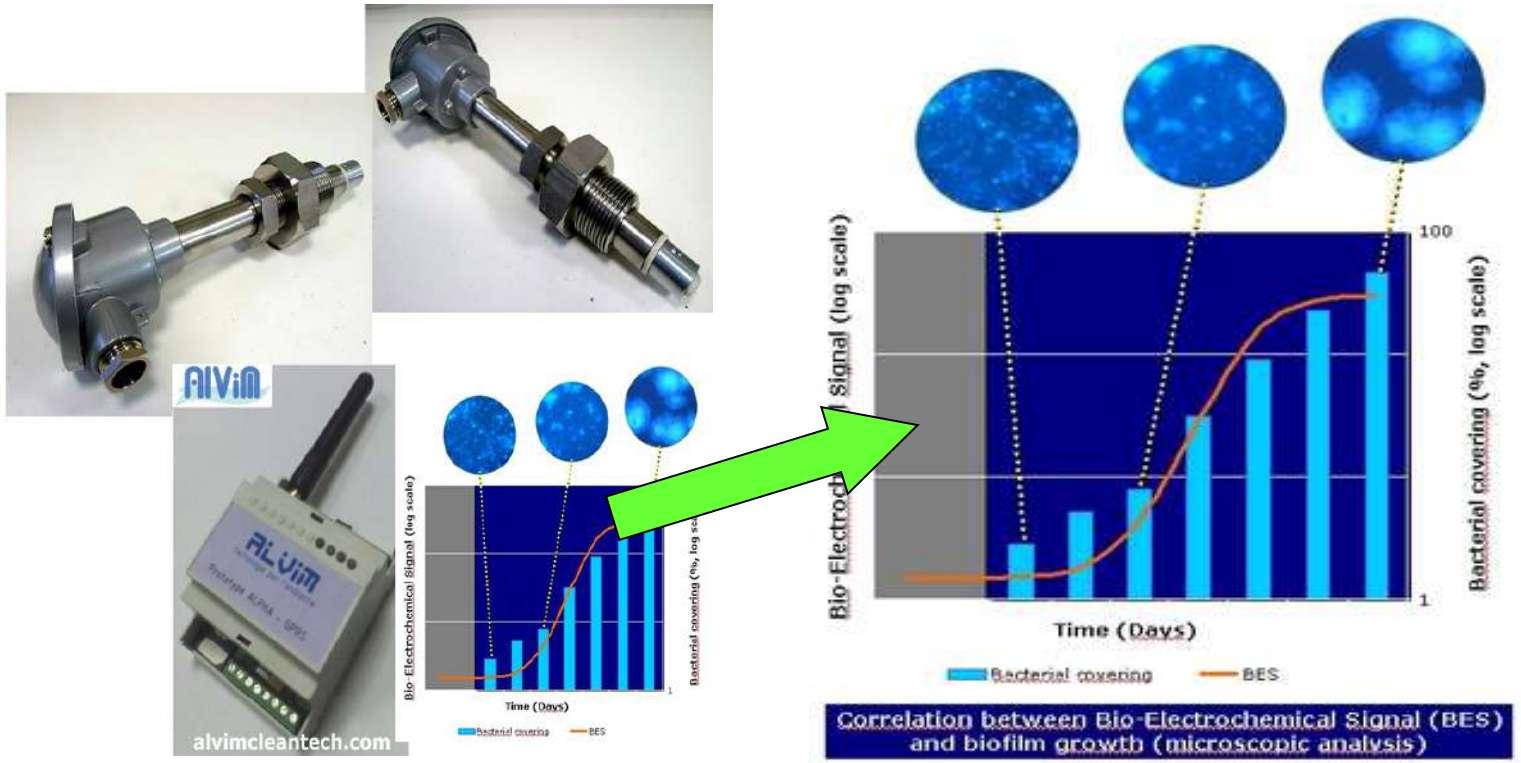
- Observation of biofilm architecture



Biofilm Detection – In-Situ Tests

Biofilm Sensors

- ALVIM - detection of biofilms using bio-electrochemical signal



Biofilm Control Strategies

Physical Treatment

- Physical removal of soil, debris, cell mass
  - Filtration
  - Scrubbing
  - Rinsing
- Increased fluid flow (minimum Re 4,000)
- Heat (> 80°C)
- Ultrasound
- Sonication

A hot and circulating purified water system
is considered self-sanitizing

Biofilm Control Strategies

Chemical Treatment

- Oxidizing Biocides
  - Chlorine, hydrogen peroxide, peracetic acid, ozone, chlorine dioxide
    - Best performance for killing microbial cells
    - Oxidizing chemicals that are highly reactive kill planktonic cells rapidly but may be consumed in the biofilm before penetrating into lower strata.
    - Slower reacting chemicals (e.g., chlorine dioxide) penetrate biofilms better achieving better kill rates.
    - React with living and non-living matter
  - Corrosion problems

Biofilm Control Strategies

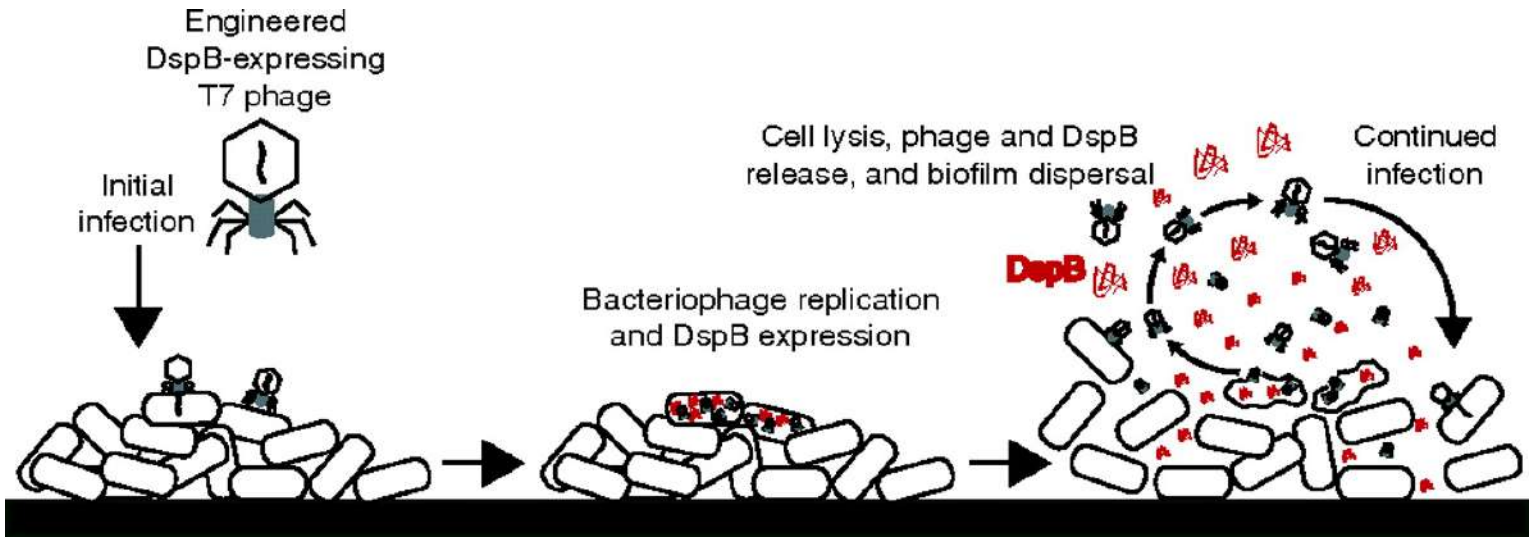
Chemical Treatment

- Non-Oxidizing Biocides
  - Isothiazolin, carbamate, quaternary ammonium compounds, phenolics
  - More targeted in their mode of action toward a particular cellular process or cell component
  - Less likely to react with non-living organic matter

Biofilm Control Strategies

Biological Treatment

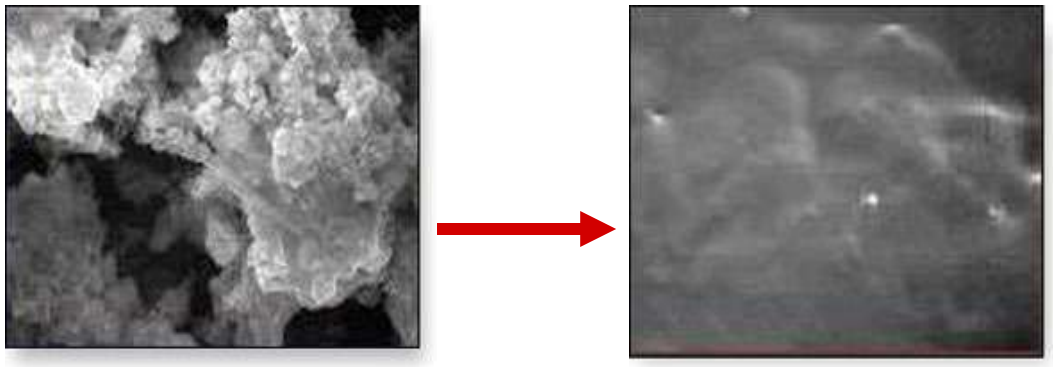
- Bacteriophage treatment targeted at specific bacteria
  - Limited applications



Biofilm Control Strategies

- Must ensure cells are **killed AND removed**
  - Many chemicals achieve sufficient reduction in Total Viable Count (TVC) but do not remove biofilm
  - Need mechanical or enzymatic action to remove biofilms
  - Chemicals must be able to penetrate the EPS matrix, kill cells, and remove dead cells

Before treatment



After treatment

Biofilm Control Strategies

- Biofilm removal agents
  - Surface active agents (surfactants)
  - Enzymes targeting the biofilm matrix
  - Chelating agents

Most effective when used in conjunction with biocides

Biofilm Prevention Strategies

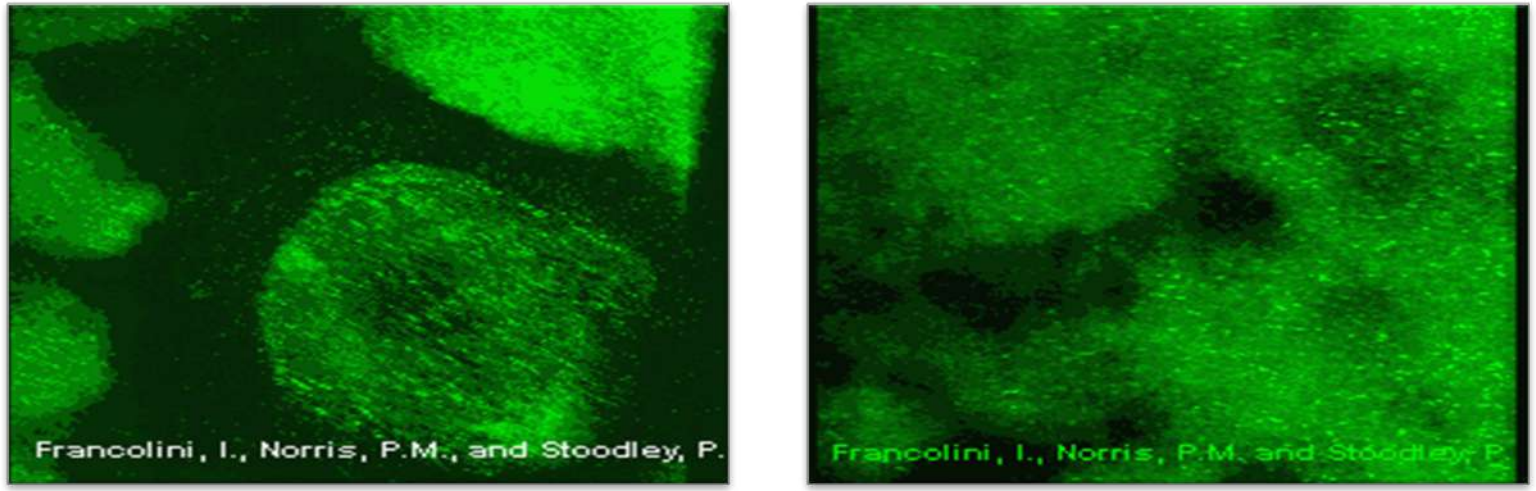
- Quorum Sensing Blockers
  - Furanones
    - **Furanone** compounds isolated from the marine macro alga *Delisea pulchra*
  - Agilyte™
    - Family of novel organic compounds derived from the sea sponge *Agelas, which* have potent anti-biofilm properties

Delisea pulchra

Agelas

Biofilm Prevention Strategies

- Anti-biofilm coatings/imbedded antimicrobials
  - Use of quorum-sensing blockers and antimicrobials
  - Short-term applications (e.g., silver-coated catheters)
  - Buildup of conditioning layer and dead cells mask effect of surface treatment over time

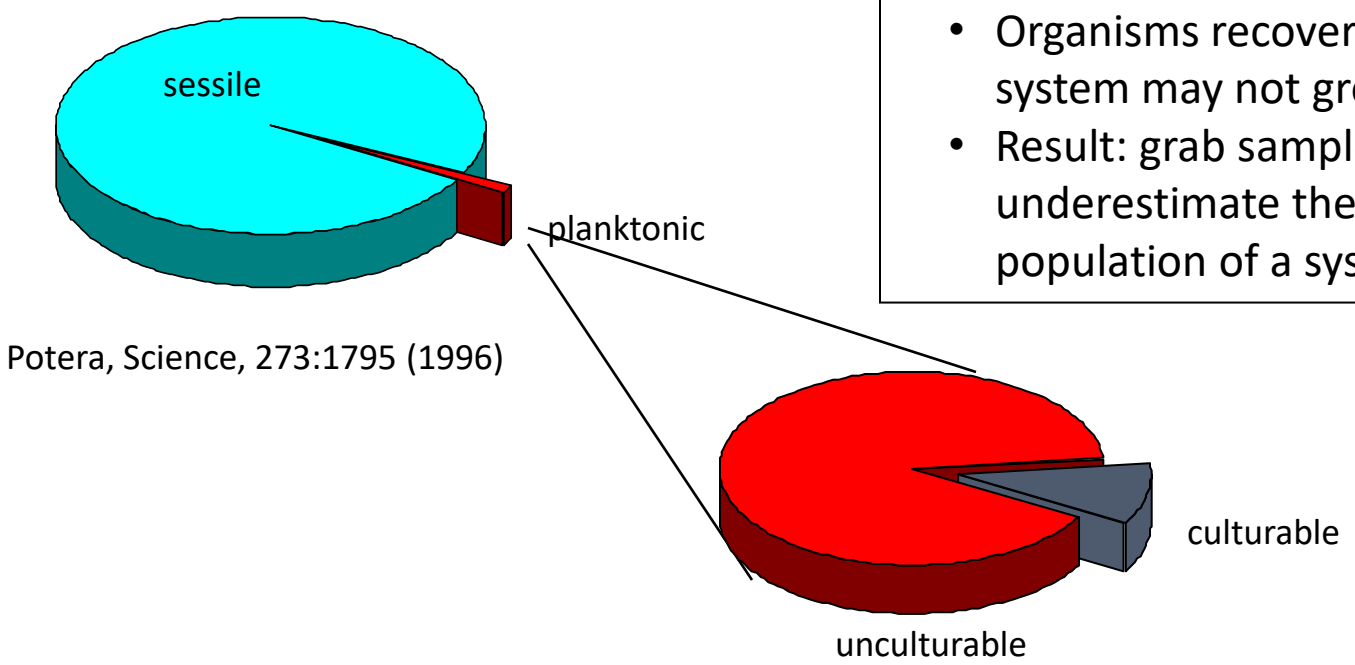


Pseudomonas aeruginosa biofilms grown on untreated polyurethane (left) and polyurethane with incorporated usnic acid (right), a natural antibiofouling agent produced by lichens.

Biofilm – The Bottom Line

- There is no material that is truly biofilm resistant
- Typical antimicrobials are ineffective against biofilms
- Biofilm cells are difficult to culture and detect using traditional microbiological methods
- Completely removing an established biofilm is very difficult

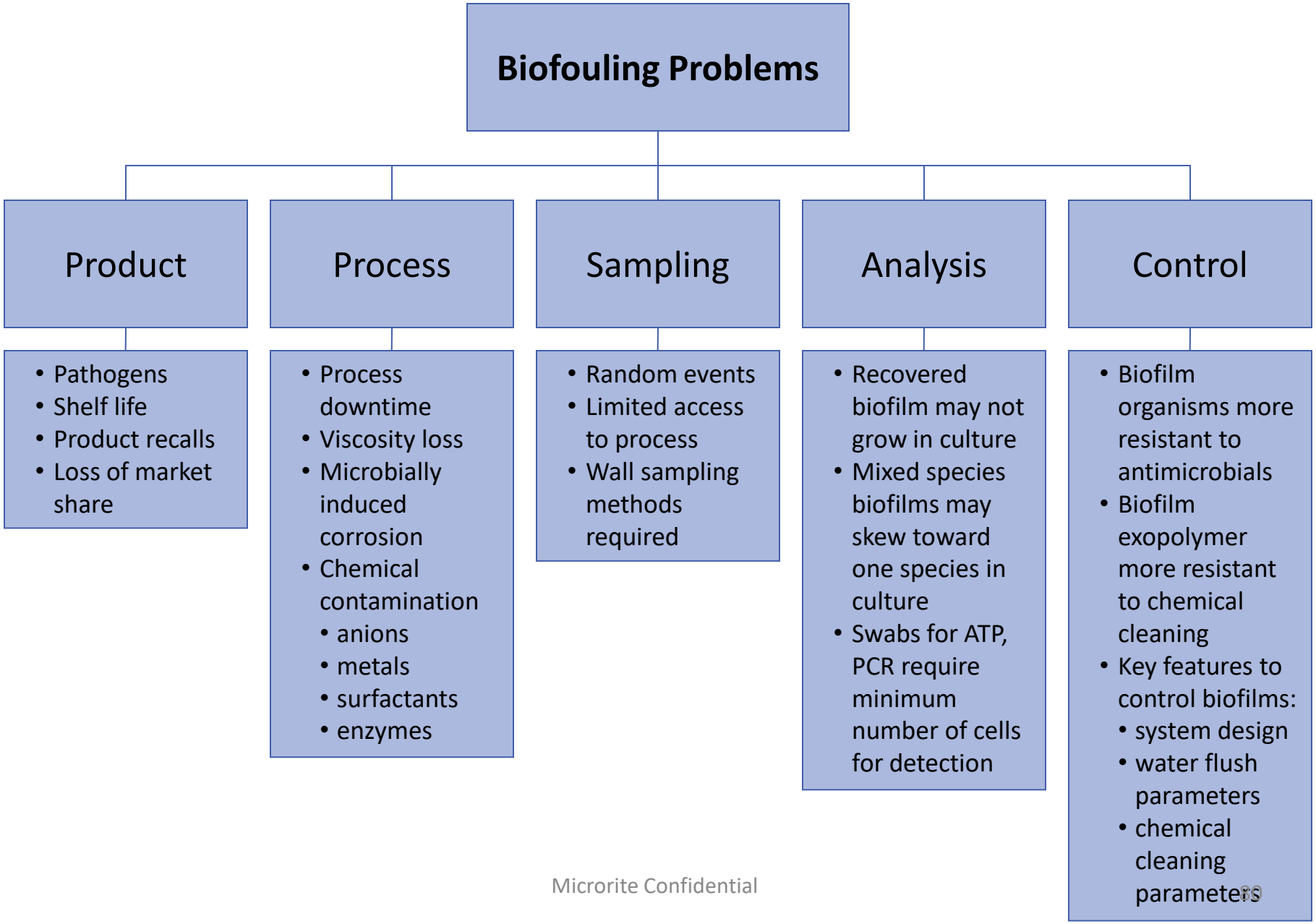
Bacteria Populations in a Pipe



Potera, Science, 273:1795 (1996)

- Sessile organisms (i.e., biofilms) are dominant form in a system
- Organisms recovered from a system may not grow in culture
- Result: grab samples may underestimate the true population of a system

White, Manual of Environmental Microbiology, 91-101 (1997)


Biofouling Problems
Product
Process
Sampling
Analysis
Control
• Pathogens
• Shelf life
• Product recalls
• Loss of market share
• Process downtime
• Viscosity loss
• Microbially induced corrosion
• Chemical contamination
• anions
• metals
• surfactants
• enzymes
• Random events
• Limited access to process
• Wall sampling methods required
• Recovered biofilm may not grow in culture
• Mixed species biofilms may skew toward one species in culture
• Swabs for ATP, PCR require minimum number of cells for detection
• Biofilm organisms more resistant to antimicrobials
• Biofilm exopolymer more resistant to chemical cleaning
• Key features to control biofilms:
• system design
• water flush parameters
• chemical cleaning parameters

Chemical Cleaning Variables

- Chemical cleaner formulation
- Concentration
- Temperature
- Order of addition
- Frequency

Properties of Biofilm

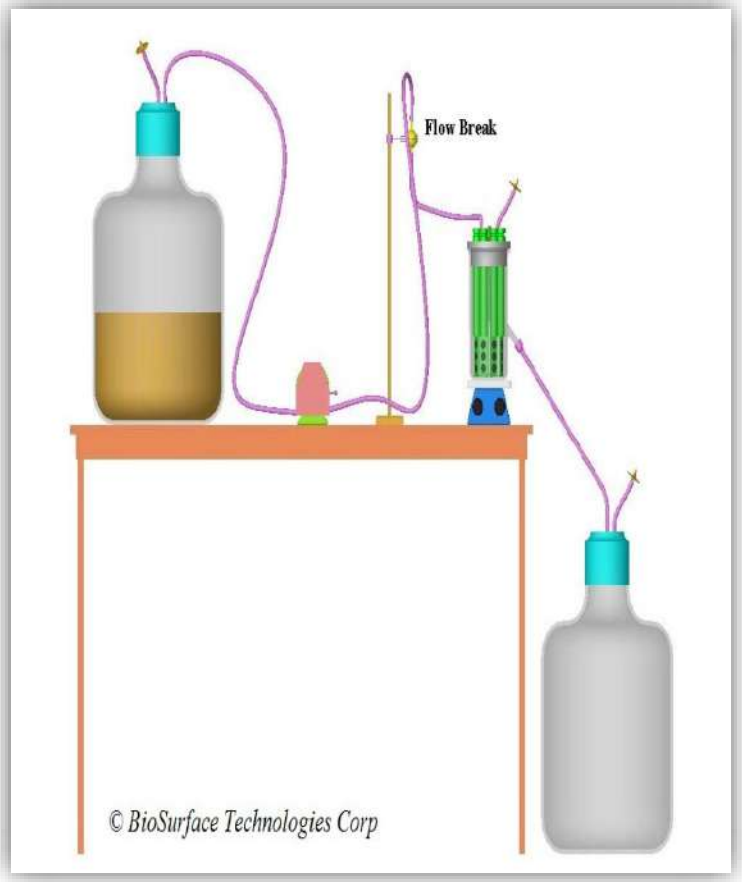
Preventing Biofilm Buildup

- Biofilms are a constant is a poorly maintained system
- The key to controlling biofilms is never to allow them to build up in the first place, is never easy to treat
- Systems not sanitized with heat are typically prone to biofilms
- Incomplete removal of the biofilm will always allow it to return to its equilibrium state and rebound after sanitization

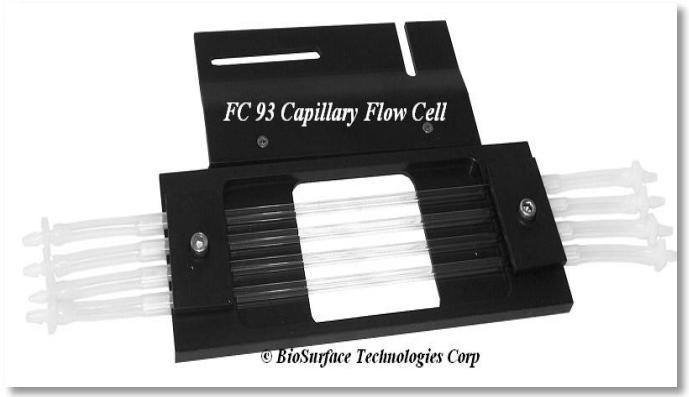
Preventing Biofilm Buildup

- Chemical and physical treatments are used to remove or destroy biofilm
- Chemical/biocides that may be used include ozone, chlorine, chlorine dioxide, hydrogen peroxide, peracetic acid and sodium hydroxide
- Chemicals added to the system must be taken out later
- Physical treatments include re-circulating hot water loops, mechanical scrubbers or scrapers, and high-pressure sprayers
- Cold-water loops are more susceptible
- The system's beginning often provides the inoculation that is the source for long-term problems

Can disinfectants be qualified using biofilm?

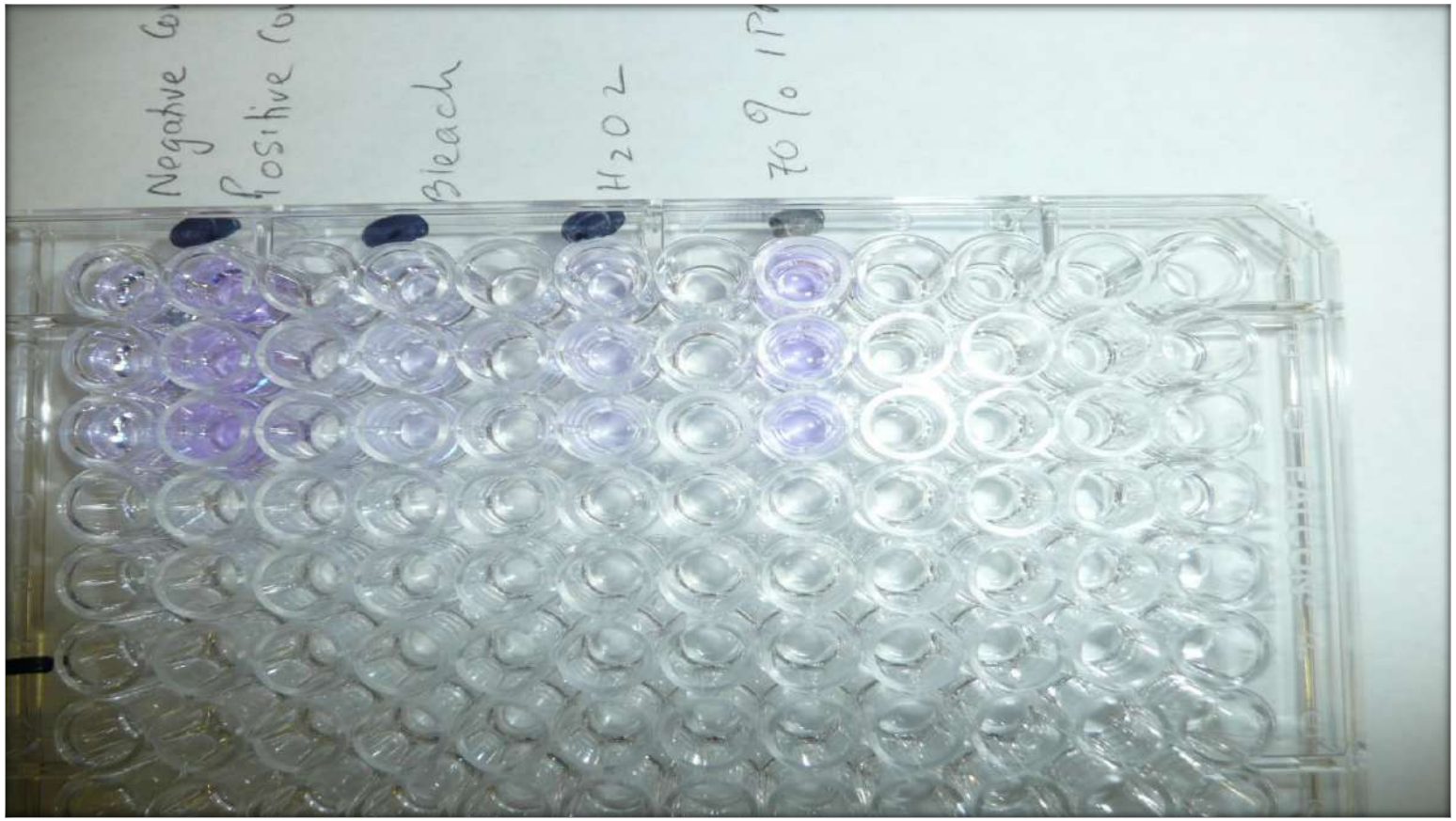


Can disinfectants be qualified using biofilm?



Disinfectant Qualification using Biofilm

Disinfectant Qualification using Biofilm



Biofilm - Conclusion

- Companies must use ***holistic and proactive*** approach to biofilm management and control
- Biofilm management is about bioburden control
- Design contamination control strategies for processes, equipment, materials, personnel, clean utilities, and facilities
    - Consider chemical, physical, and procedural controls
    - Use new technologies (e.g., alternative methods) for early detection of microbial contamination and biofilm formation.

Biofilm – Conclusion

- Companies using biofilm reactors to grow biofilms in the laboratory for antibiofouling studies
  - BioSurface Technologies Corporation (www.biofilms.biz)
  - ASTM methods available for some devices

- PDA *Bioburden and Biofilm Management Task Force* will issue a Technical Report (~ July 2013)
  - Best Practices for the Detection and Control of Bioburden, Including Biofilms, in Pharmaceutical Sterile Processing Operations

Acknowledgement: Pictures and videos used for this presentation have been contributed by Mark Fornalik and those from Microrite Library

Viable but Non Culturable Organisms

VBNC

Microorganisms are exposed to a wide variety of stress factors in their natural environments.

Under these stressful conditions, they move into a viable but nonculturable (VBNC) state to survive and maintain the vitality.

At VBNC state, microorganisms cannot be detected by traditional laboratory methods, but they can be revived under appropriate conditions.

Therefore, VBNC organisms cause serious food safety and public health problems.

To date, it has been determined that more than 100 microorganism species have entered the VBNC state through many chemical and physical factors.

During the last four decades, dating from the initial detection of the VBNC condition, new approaches have been developed for the induction, detection, molecular mechanisms, and resuscitation of VBNC cells.

VBNC

As a unique microbial response to adverse circumstances, the viable but nonculturable (VBNC) state is characterized by the loss of culturability of microbial cells on/in nutrient media that normally support their growth, while maintaining metabolic activity.

Through disinfection, sanitation, hygiene practices, biocides and antibiotics, we have considerably improved in our ongoing battle against pathogenic bacteria.

However, with our increasing knowledge about the complex bacterial lifestyles and cycles and their plethora of defense mechanisms, it is clear that the fight is far from over.

Now it is clear that the VBNC state constitutes an important reservoir of pathogens in the environment.

The medical implications of this fact are numerous.

For example, it appears that the ‘latent’ or the ‘dormant’ phase of *Mycobacterium tuberculosis* infections represents the VBNC state in this pathogen and that the recurrence of tuberculosis years after a person was thought to be tuberculosis free is due to this phenomenon.

The list of pathogenic bacteria that have adopted this lifestyle as a means of survival includes not only those that infect humans but also those that infect such diverse animals, corals, and sea urchins and others.

Many plant pathogenic bacteria entering this state have also been described.

List of pathogens is ever increasing!

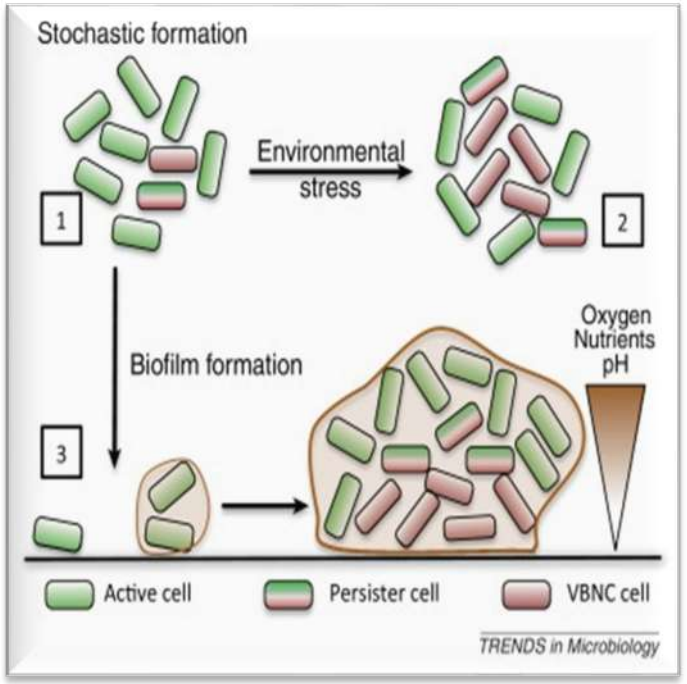
What It Tells You, What It Does Not

"Viable but non-culturable cells (VBNC) are defined as live bacteria, but which do not either grow or divide.

Such bacteria cannot be cultivated on conventional media (they do not form colonies on solid media, they do not change broth appearance), but their existence can be proved using other methods."

Ref:
https://pubmed.ncbi.nlm.nih.gov/21038700/

STOCHASTIC is *random*; specifically : involving a random variable
Persisters are dormant variants of regular *cells* that form stochastically in microbial populations and are highly tolerant to antibiotics.



"The viable but nonculturable (VBNC) state is a unique survival strategy of many bacteria in the environment in response to adverse environmental conditions. VBNC bacteria cannot be cultured on routine microbiological media, but they remain viable and retain virulence."

https://www.hindawi.com/journals/isrn/2013/703813/

VNBC?

The Significance and Detection of VBNC Microorganisms

American Pharmaceutical Review

Friday, June 1, 2007

https://www.americanpharmaceuticalreview.com/Featured-Articles/113051-The-Significance-and-Detection-of-VBNC-Microorganisms/

Table 1: Bacteria that can exist in the VBNC state

Aeromonas salmonicida
Agrobacterium tumefaciens
Campylobacter jejuni
Enterobacter aerogenes
Enterococcus faecalis
Escherichia coli
Helicobacter pylori
Klebsiella pneumoniae
Lactobacillus plantarum
Legionella pneumophila
Micrococcus luteus
M. varians
Pasteurella piscida
Pseudomonas aeruginosa
P. fluorescens
P.putida
P.syringae
Salmonella enteritidis
S. typhimurium
Shigella dysenteriae
S. flexneri
S. sonnei
Vibrio anguillarum
V. campbellii
V. cholerae
V. fischeri
V. Harveyi
V. mimicus
V. natriegens
V. parahaemolyticus
V. proteolytica
V. vulnificus (biotypes 1 and 2)

The list is ever increasing

Aeromonas hydrophila	*Mycobacterium tuberculosis*	*V. shiloi*
Helicobacter pylori	*V. anguillarum*	*E. faecium*
Serratia marcescens	*C. lari*	*R. meliloti*
Klebsiella aerogenes	*M. smegmatis*	*V. vulnificus (types 1 & 2)*
Shigella dysenteriae	*V. campbellii*	*Erwinia amylovora*
Agrobacterium tumefaciens	*Cytophaga allerginae*	*Salmonella enterica*
K. pneumoniae	*Pasteurella piscicida*	*Xanthomonas campestris*
S. flexneri	*V. cholerae*	*Escherichia coli (including EHEC)*
Burkholderia cepacia	*Enterobacter aerogenes*	*S. typhi*
K. planticola	*Pseudomonas aeruginosa*	*X. axonopodis pv. citri*
S. sonnei	*V. harveyi*	*Francisella tularensis*
B. pseudomallei	*E. cloacae*	*S. typhimurium*
Legionella pneumophila	*P. syringae V. mimicus*	*V. shiloi*
Streptococcus faecalis	*Enterococcus faecalis*	*E. faecium*
Campylobacter coli	*Ralstonia solanacearum*	*R. meliloti*
Listeria monocytogenes	*V. parahaemolyticus*	*V. vulnificus (types 1 & 2)*
Vibrio alginolyticus	*E. hirae*	*Erwinia amylovora*
C. jejuni	*Rhizobium leguminosarum*	*Salmonella enterica*

And more are being discovered

Inducers of the VBNC State

A list of factors, both chemical and environmental, which have been reported to induce the VBNC state, are varied and numerous including:

- Nutrient starvation
- Incubation outside the normal temperature range of
- Elevated or lowered osmotic concentrations
- Oxygen concentrations commonly used food preservatives
- Heavy metals

A common response to such stresses by bacterial cells is their ultimate
inability to develop into colonies on routine culture media

Antibiotic resistance of VBNC cells

One of the interesting and significant consequences of entry of pathogens into the VBNC state includes its effects on antibiotic resistance when pathogens are in this state (often in biofilms).

It seems likely that, because VBNC cells demonstrate such low metabolic activity, they effectively become resistant to antibiotics, and yet are able to resuscitate and reinitiate infections.

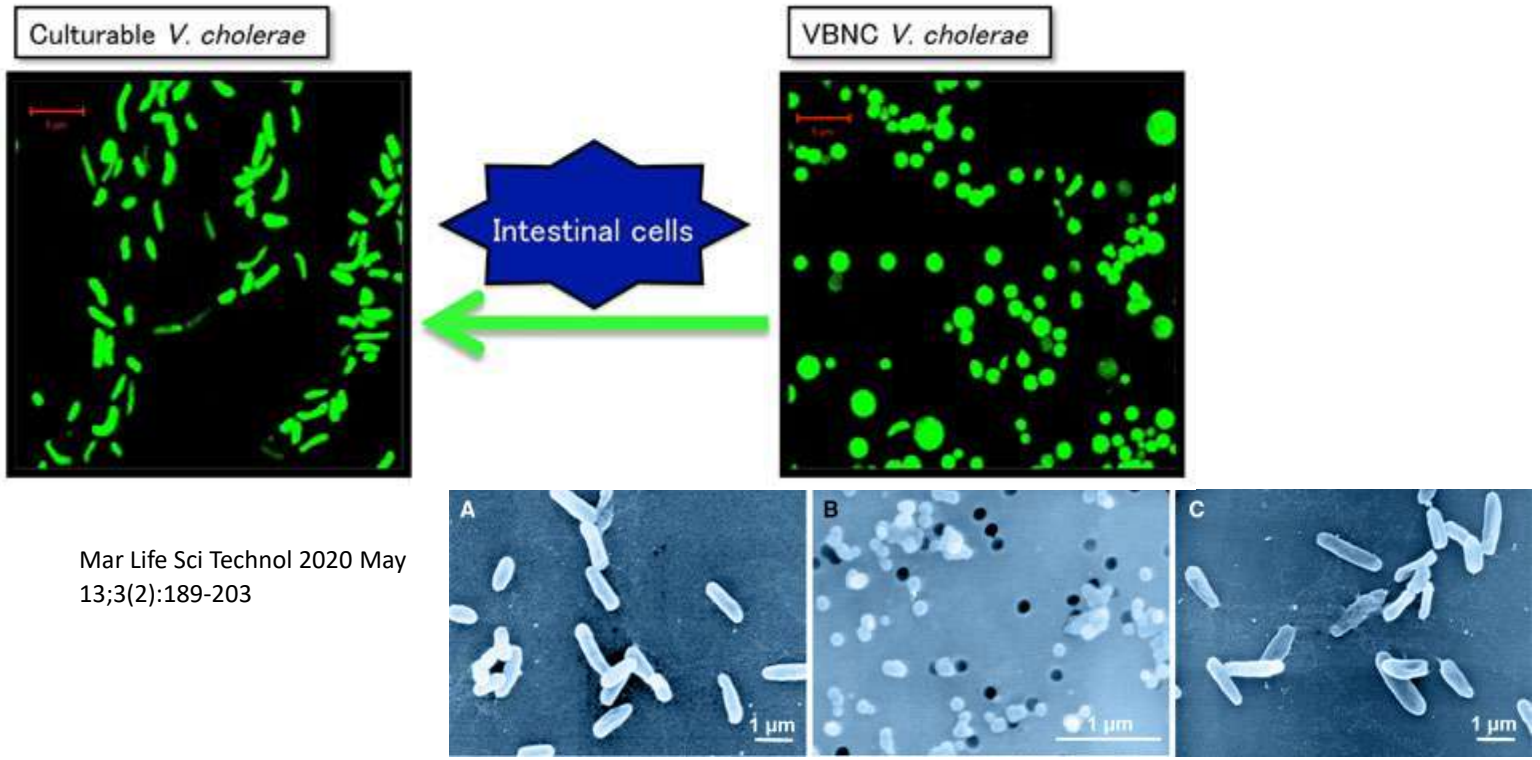
For example, antibiotic resistant VBNC cells of *Haemophilus influenzae* present in biofilm are able to initiate chronic ear infections.

Another example is *H. pylori*, which produces gastric and duodenal ulcers, which are among the most widespread and common syndromes in the world and is shown to rapidly enter the VBNC.

The recurrent urinary tract infections suffered by many individuals are thus likely a result of cells in this temporarily dormant state, which are able to resist antibiotic treatment and resuscitate back to the metabolically active state.

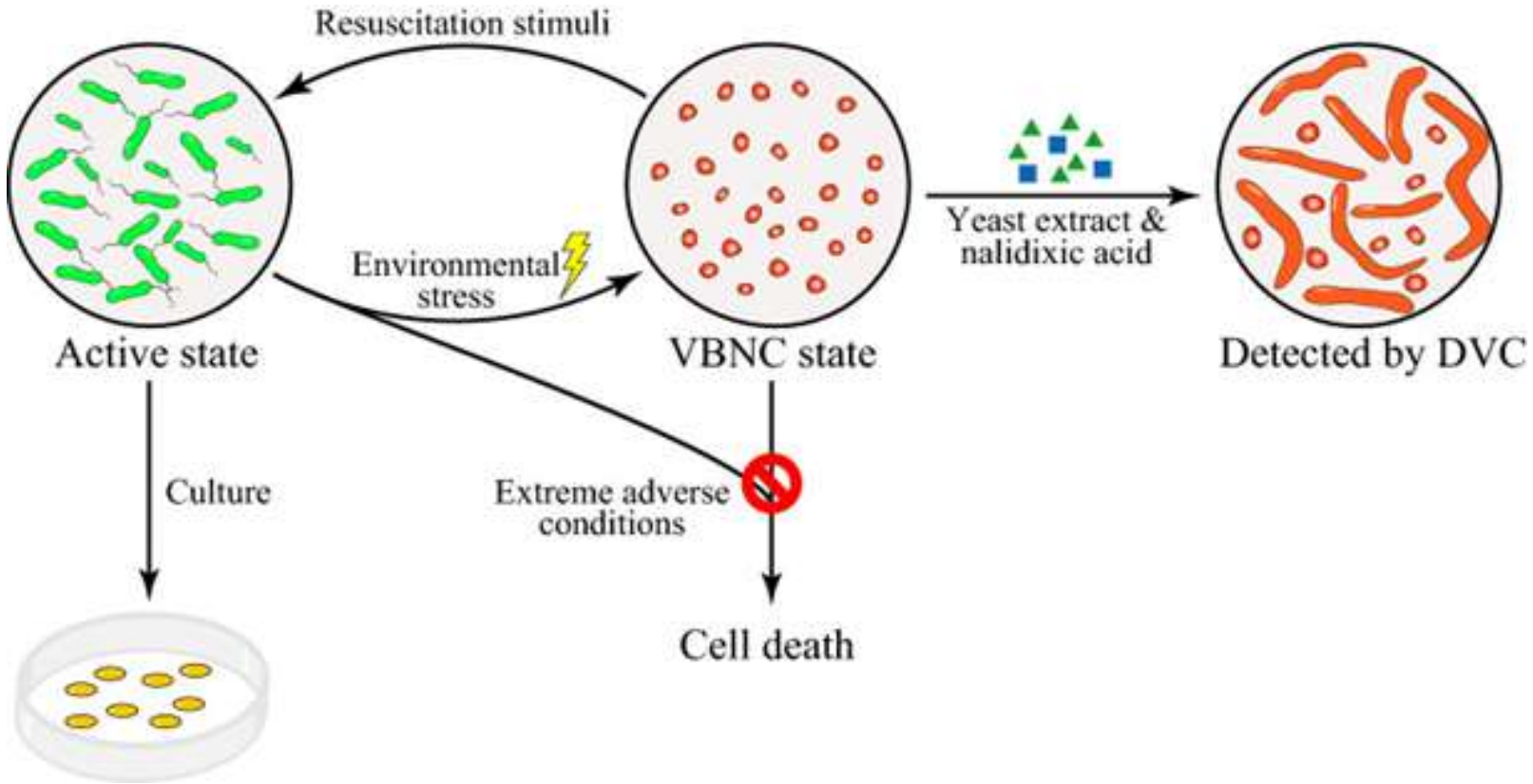
Survival in VBNC State

Examples:
Pseudomonas fluorescens cells can remain in this state in soil for over a year
Vibrio fluvialis could be resuscitated 6 years after becoming VBNC in marine sediment



Mar Life Sci Technol 2020 May 13;3(2):189-203

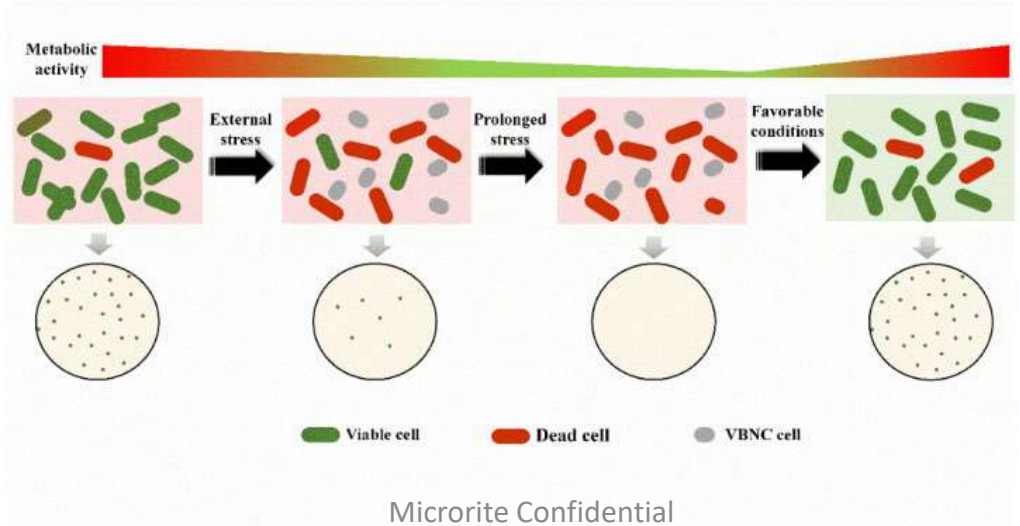
Resuscitation of cells from the VBNC state



Resuscitation of cells from the VBNC state

The VBNC state can only be a significant means of survival if the cells are able to increase metabolic activity and again become culturable.

Proving that true resuscitation of cells from the VBNC state occurs (as opposed to simple regrowth of a few undetected and culturable cells present in the VBNC population) has been problematic and a source of much of the disagreement concerning the validity of a VBNC state among bacteria.



Why do cells enter the VBNC state?

It is suggested that one aspect of the VBNC state likely involves H_2O_2, either following its production by the cells when plated onto solid media, or its natural presence in solid media, coupled with an inability of the cells to detoxify this lethal metabolite.

If cells entering into the VBNC state were plated onto conventional laboratory media (e.g. heart infusion agar, etc) supplemented with peroxide-neutralizing agents (e.g. catalase), considerably enhanced culturability was seen.

Low-temperature incubation may also result in cells that, due to this cold shock response, are nonculturable and yet remain viable.

Effect of Antimicrobials

The VBNC state of several pathogens such as *E. faecalis, H.pylori,* and *Haemophilus influenza* has been found to be resistant for several antimicrobials.

S. aureus presents as biofilm can cause recurrent infection in the VBNC state in the presence of vancomycin and other antibiotics used for treatment.

Mycobacterium tuberculosis is multi-drug resistance and may prolong the infection and chemotherapy for longtime.

Conditions that induce and reverse VBNC State

Several factors such as antibiotic, pressure, high/low temperature, starvation, chlorination, change in the pH, and oxygen stress can induce VBNC state of any bacteria.

Favorable growth conditions with a source of energy and an ideal stoichiometric ratio of carbon to inorganic elements can reverse the VBNC state. This phenomenon is widely known as resuscitation.

The most notable advance in resuscitating VBNC bacteria is the discovery of resuscitation-promoting factor (Rpf), which is a bacterial cytokines found in both Gram-positive and Gram-negative organisms.

VBNC state is a survival strategy adopted by the bacteria, which has important implication in several fields, including environmental monitoring, food technology, and infectious disease management; and hence it is important to investigate the association of bacterial pathogens under VBNC state and the water/foodborne outbreaks.

Methods for the detection of VBNC bacteria

- Acridine orange staining
- Fluorescent antibody-direct viable
- count (DFA-DVC)
- Elongation of cells in the presence of nalidixic acid or ciprofloxacin
- CTC and DAPI double staining
- Respiratory activity
- Metabolic activity
- Quantitative PCR (qPCR)
- RT-PCR Quantification of 16SrRNA
- DVC-FISH Measure of RNA
- MALDI-TOF/MS Identification of differentially expressed proteins
- Fluorescent viability staining
- Identification based on intracellular esterases
- RING-FISH Detection of individual genes
- Biosensor Detection of beta-d-glucuronidase
- SELEX technique DNA aptamer-based viability detection
- Bacteriophages

There is an urgent need for further research involving a combination of cultural and molecular approaches to understand the phenomenon, as the formation of VBNC state in bacteria and its resuscitation pose a great threat to public health.

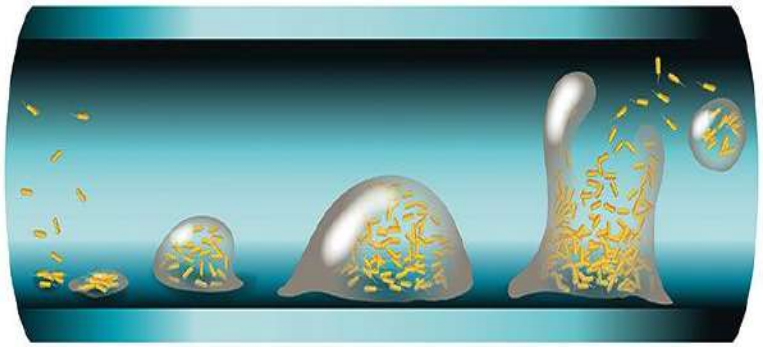
For the same reason, further improvement of the detection methods involving fluorescent reporter dyes, microfluidic, micro electromechanical systems, and time-lapse fluorescence microscopy remains an urgent necessity.

CASE STUDY : Non-tuberculous Mycobacteria in Pharmaceutical Water System

Discussion

- Adhesion and biofilm formation abilities of various Gram-negative bacteria is observed in pharmaceutical water systems; the presence of non-tuberculous mycobacteria is not commonly discussed.

- The presence of non-tuberculous mycobacteria is known in hospital water systems and dreaded in medical devices because of its capability to circumvent certain disinfectants.



CASE STUDY : Non-tuberculous Mycobacteria in Pharmaceutical Water System

Discussion

- Non-tuberculous mycobacteria (NTM) have been found to be ubiquitous in the environment and have been isolated from numerous water sources, including wastewater, surface water, recreational water, ground water and tap water.

- Piped water supplies are readily colonized by mycobacteria thus biofilm may serve as a reservoir for these opportunistic pathogens. NTM are common environmental organisms and are recognized as being difficult to remove from water systems.

CASE STUDY : Non-tuberculous Mycobacteria in Pharmaceutical Water System

Discussion

- Since the water system in this case stood idle for an extended period, biofilm formation was inevitable.

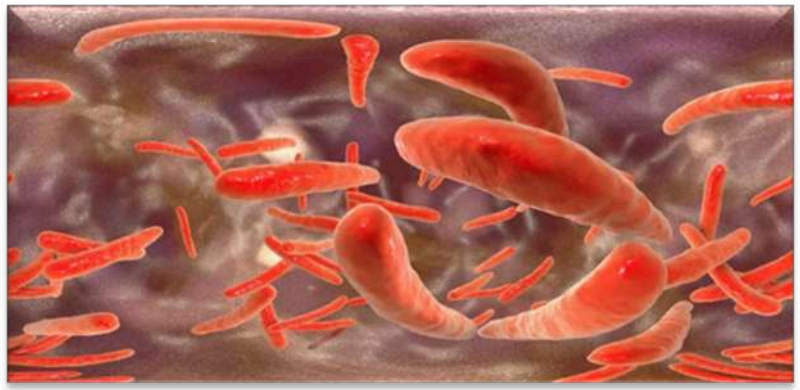
- Additionally, as these organisms require longer incubation periods, they were not detected until they had the opportunity to present in media fill samples where incubation time is longer than that for routine environmental monitoring and water sampling.

CASE STUDY 1: NONTUBERCULOUS MYCOBACTERIA IN WATER PHARMACEUTICAL WATER SYSTEM

Lessons learned

- If water systems are not designed, maintained and tested using adequate methods for detecting contamination, biofilm formation is certain and organisms that pass through 0.2 micron filters may be present in the process water and yet not detected due to inadequate growth media or incubation- Pleomorphism.

- Most water borne organisms are pleomorphic in nature and a few such mycobacteria and some other Gram-negative bacteria are known to pass through filters due to their minuscule size



Concluding remarks

There are many species that are known to exist in nature but are not culturable in the lab, the so-called “viable but nonculturable” (VBNC). There is another group of bacterial species that are culturable but grow very slowly. The exact role of the VBNC state in bacteria is yet to be elucidated.

It is likely that its role and significance differ from bacterium to bacterium.

Most investigators believe it to be a response to certain environmental stresses that allows the cell’s survival.

In some, for example, entry into this dormancy state appears to be simply one aspect of ‘cold shock response’ that bacteria exhibit with non culturability being an ‘artifact’ of the sensitivity of such cells to the toxic peroxide present in
laboratory media.

It has been recently suggested that dormancy, and ‘waking up’ from this state, could be a method analogous to ‘sending out scouts’ to ‘test the environment’
for its suitability for growth of the entire population.

In this scenario, if the resuscitating cells ‘detect’ that the previously stressful/adverse environment is now growth-permissive, they
would signal the remaining cells to resuscitate.

Questions?

Refreshments Break

Please return to your seats in 15 minutes

8/17/2023

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Speaker Introduction

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Christian Scheuermann

Global Technical Services Manager at Charles River Laboratories - Microbial Solutions

Christian Scheuermann has more than 20 years of microbiology experience from both academic and industrial environments. He earned his Master's degree in Biology at the University of Würzburg with additional studies at the Universities of Frankfurt and Naples (Italy) and at the Max-Planck-Institute.

With regards to pharmaceutical microbiology, Christian is specialized in the fields of microbial quality control, microbial identification and phylogenetics.

He is currently working at Charles River Laboratories, Microbial Solutions division as Global Technical Services Manager.

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Risk Mitigation with Modern Technologies

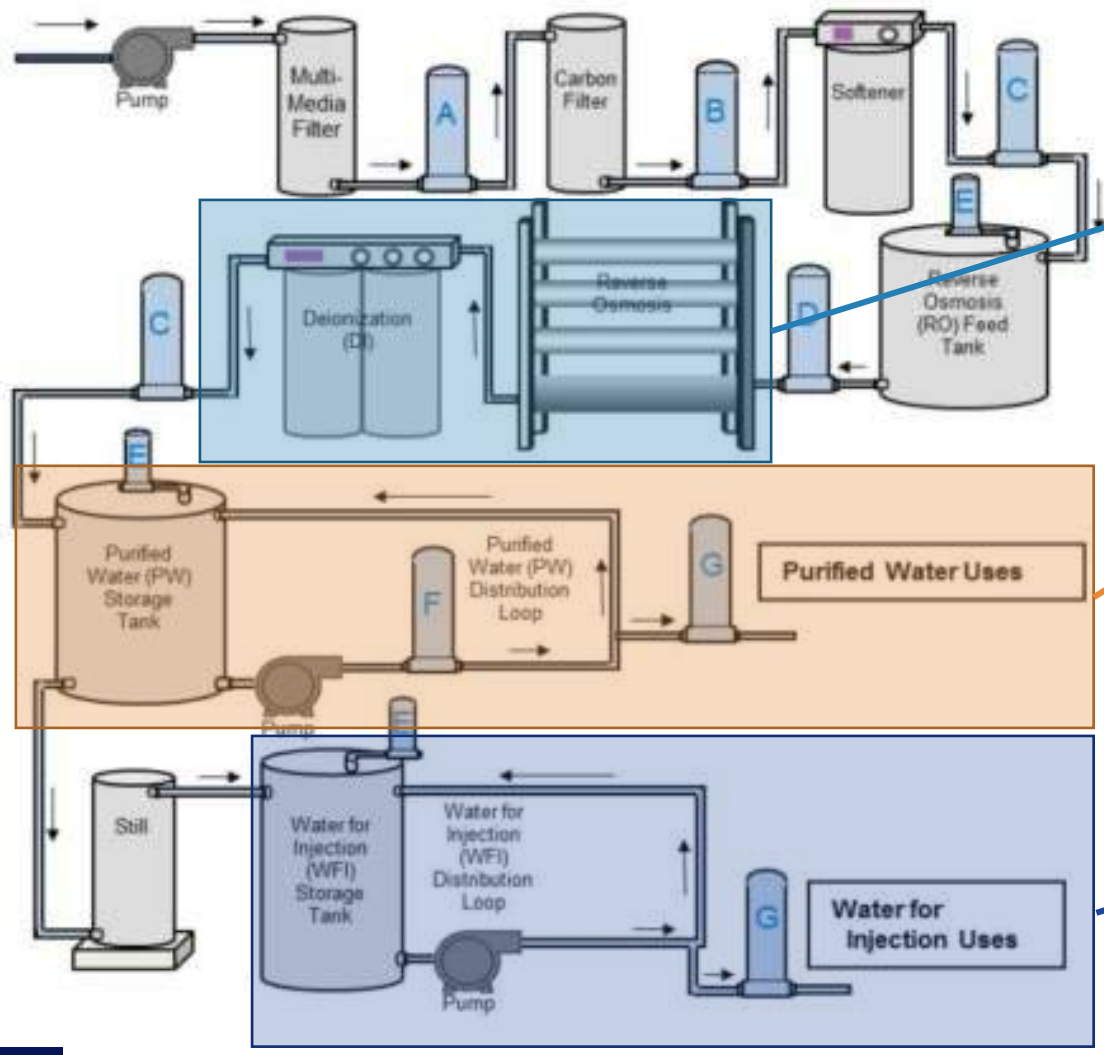
Focus: Water, Biofilms and VBNCs

Christian Scheuermann
Global Technical Services Manager
Charles River Microbial Solutions

08/17/2023

WFI: Microbial Expectations

WFI as a Critical Utility



Microbiological requirements

Deionized water	
Bioburden limit	NA
Endotoxin limit	NA

Purified water (EP GM0008 / USP 1231)	
Bioburden limit	**<100cfu/mL (10000cfu/100ml)**
Endotoxin limit	NA

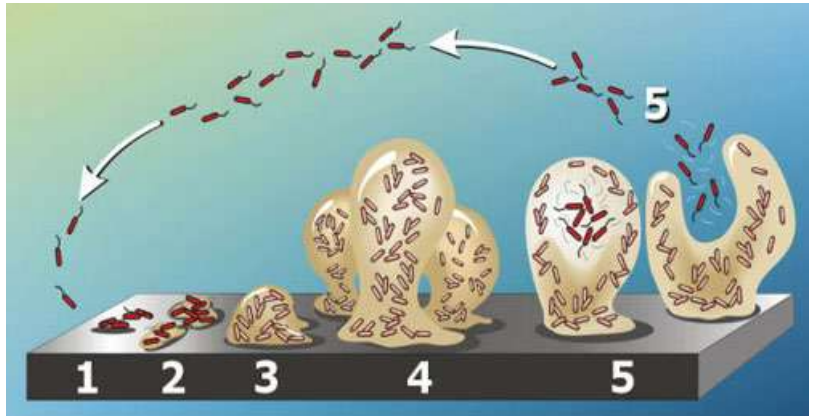
WFI(EP GM0169 / USP 1231)	
Bioburden limit	**<10cfu/100mL (not sterile)**
Endotoxin limit	<0.25EU/mL

Water Quality increase cascade

Annex 1 – Chapter 6 Utilities – Water systems 6.7.

Due to WFI systems criticality and that WFI requirements accept a certain amount of bioburden coming from the systems that supply water to them, these WFI systems must be:

MONITORED | **QUALIFIED**



MINIMIZE RISK microbial contamination/proliferation and pyrogens

+

PREVENT formation of BIOFILMS

ENSURE A reliable source of water of an appropriate quality.

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USP <1231> and Microbial Control of Waters

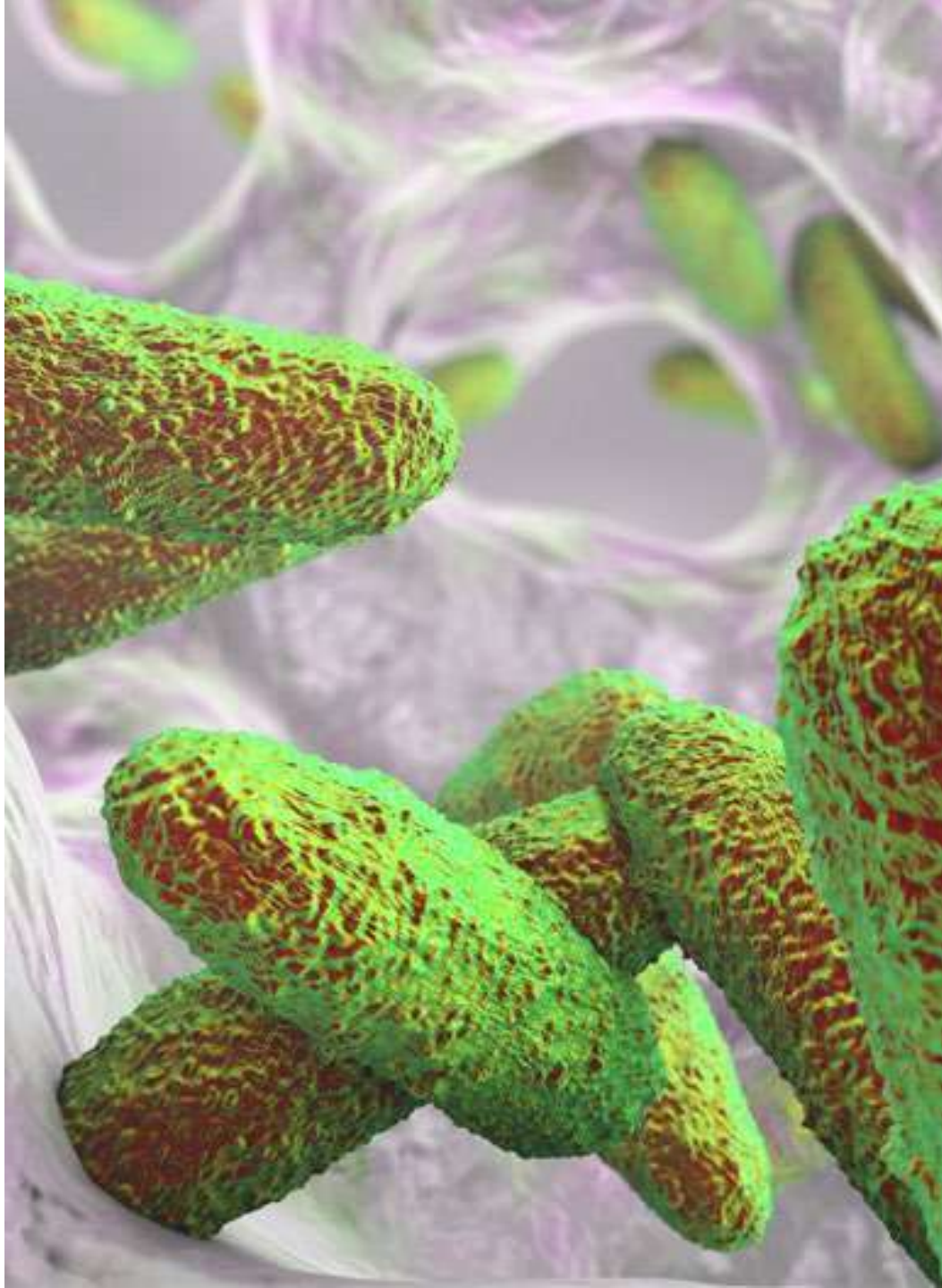
USP <1231> Microbial Considerations of Waters used in Pharmaceutical Manufacturing and Testing –
General Information Chapter

- Exogenous sources of microbial contamination
  - + Source or feed water
  - + Soil or humans (faulty aseptic technique during sampling or testing)
- Endogenous sources of microbial contamination
  - + Poor operation/maintenance of water systems
  - + Biofilm formation
- Mainly aquatic Gram-negative bacteria are found in water systems
  - + Detection of non-aquatic microorganisms may indicate sampling/texting contamination or component failure, which should trigger investigation/remediation

IDENTIFICATION OF MICROORGANISMS

Identifying the isolates recovered from water monitoring methods may be important in instances where specific waterborne microorganisms may be detrimental to the products or processes in which the water is used. Microorganism information such as this may also be useful when identifying the source of microbial contamination in a product or process. Often a

(Regulatory) Recommendations for Biofilm Control



Microbial Monitoring of Critical Utilities - Water systems

WFI CCS - SAMPLING PLAN

DAILY

6.13. Regular ongoing microbial monitoring of water systems should be performed.

i. All points of use, at a specified interval (QRM).
ii. A sample from the point at the end of the distribution loop each day that the water is used.

QUALIFICATION

INITIAL

PERIODIC

6.8. Water systems should be qualified to maintain the appropriate levels of microbial control, taking seasonal variation into account.

Biofilms in Guidelines – EMA Q & A

PART II - BIOFILMS AND CONTROL STRATEGIES

2. What approach should be taken to maintain control over systems which can be affected by biofilms?

A control strategy should be developed to assess the risks associated with the current manufacturing processes and to determine acceptability of existing control measures. The effectiveness of the sampling and testing regimes employed at the site should also be critically assessed in conjunction with the development of a control strategy.

3. What is a control strategy in the context of biofilm and contamination control?

A control strategy should take account of the design of the process, the mechanisms required to be put in place to control and ultimately prevent or minimise the risk of contamination.

Such a strategy requires the following thorough process knowledge and understanding taking account of all aspects of contamination control and prevention, including:

- Design
- Water system qualification
- Personnel qualification/training
- Raw Materials, e.g.
- Monitoring systems (qualification/calibration) used in the control strategy
- Preventative maintenance to a standard that will not add significant risk from a contamination view point
- Control strategy including in-process controls applied to
- Robust QMS
  - Deviation handling
  - Root cause analysis (investigations)

Contamination control and steps taken to minimise the risk of contamination are a series of successive linked events/measures. Quality Risk Management tools along with scientific judgement can be applied in determining critical control points.

A contamination control strategy would integrate all of these measures to ensure a more comprehensive approach is taken with respect to prevention and control of microbiological contamination.

Such a strategy should lead to the introduction of a control programme which is an iterative process taking into account all information throughout the lifecycle of the products and processes.

https://www.ema.europa.eu/en/documents/other/questions-answers-production-water-injections-non-distillation-methods-reverse-osmosis-biofilms_en.pdf

Biofilms in Guidelines - EMA

PART II - BIOFILMS AND CONTROL STRATEGIES

6. Are there any additional measures which should be considered in order to increase the probability of detecting the presence of biofilms?

A robust sampling plan is a requirement. Such a sampling plan forms part of the assessment of the effectiveness of the control strategy employed to minimise such risks of biofilm and general contamination issues. Each potential source of contamination should be incorporated into such a sampling regime. Ongoing evaluation to determine the appearance of an adverse trend should be performed, however, the seasonal variation that occurs can only be determined during the annual trend assessment. The effectiveness of an environmental monitoring programme should be formally assessed at minimum on an annual basis.

Sampling programmes for water systems should take account of the quality of the water supply to the system as well as assessing points throughout water generation. Water quality is best assessed through a pre-determined, systematic approach. The loop return should be sampled each day of use of the system in order to provide additional assurance of the quality of water utilised in the manufacturing processes. All points should be sampled on a rotational basis to ensure that the entire system user points are sampled at least once per week.

Routine identification of contaminants isolated during monitoring activities is critical in order to ascertain if there is any shift or change in the flora present within a facility or if certain specific species become more prevalent.

Use of more sensitive endotoxin detection methods should also be taken into account. Alert levels should be set based on the capability of the system and any change or adverse trend should be appropriately investigated.

The frequency of trend analysis and use of trend data is critical. The use of rapid microbiological test methods and systems should be considered in order to improve or increase the probability of early detection and allow timely action to be taken.

https://www.ema.europa.eu/en/documents/other/questions-answers-production-water-injections-non-distillation-methods-reverse-osmosis-biofilms_en.pdf

Endotoxin Testing as a Detection Method for Bacterial Biofilms

American Pharmaceutical Review Article | Dr. Tim Sandle

Article Summary: Endotoxin testing can play a role in the earlier detection of biofilms than is possible using conventional bioburden tests. This is on the assumption, albeit one supported by most literature, that much of the bacterial contamination of water systems, and to an extent medical implants, is by Gram-negative bacteria, which on lysis would release endotoxin.

Endotoxin Testing as a Detection Method for Bacterial Biofilms | American Pharmaceutical Review - The Review of American Pharmaceutical Business & Technology

Methods to assess Microbiological Quality for WFI Systems

	Bioburden	Endotoxin testing
Analyte	Viable microorganisms	Non-viable, high resistant and stable microorganisms particles
Limit of detection	1CFU/200mL	Sensitivity up to 0,005EU/mL parts per trillion (ppt; 10^{-12})
Specificity	All microorganisms that are able to grow in the media used (R2A?)	High specificity to gram-negative bacteria

Biofilms: The hidden Detection and Biodiversity Challenge

Goal: Early Biofilm Detection

To minimize production impact we need an EARLY BIOFILM DETECTION whilst complying with Annex 1 requirements:

- LESS spreading => LESS BIOFILM focus => more easy disinfection
- LESS batches impacted

To keep critical utilities under the required quality, CCS requires:

- an effective sampling plan
- and a test method that must be specific and sensitive enough to optimize contaminant detection

Methods to assess microbiological quality for WFI systems:

CLASSICAL BIOBURDEN METHOD

ENDOTOXIN TESTING AS AN EARLY DETECTION METHOD FOR BACTERIAL BIOFILMS

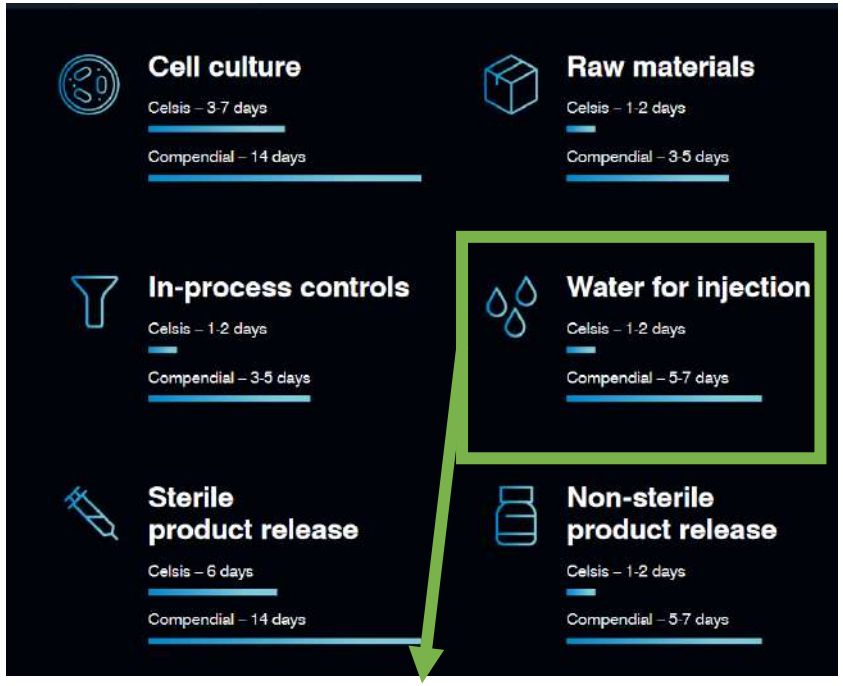
Rapid Bioburden Detection for Water: An ATP based Rapid Microbial Method

Traditional quality control testing methods require 3–7 incubation days for bioburden or microbial limits (MLT) and, for sterility, more than 14 days of non-value-add incubation at every point of microbiological testing.

Celsis® rapid microbiology delivers results in just 18–24 hours for bioburden and microbial limits and just 6 days for sterility testing results, less than half the time of that required by traditional methods.

The obvious benefits are:

- Shortened production cycles,
- Decreased working capital requirements,
- Reduced lab waste to improve sustainability,
- Faster response to contamination and shorter investigations,
- Reduced inventory requirements and safety stock.



An appropriate ATP rapid method could not only prove that the WFI is within spec, but also *most potentially* indicate the presence of a biofilm due to free floating ATP within in the water system.

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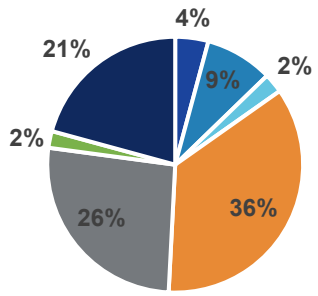
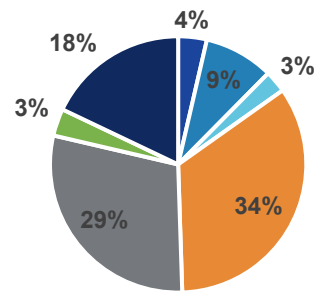
Relative Distribution of most commonly isolated Biofilm Genera per Global Region

APAC, n = 2745



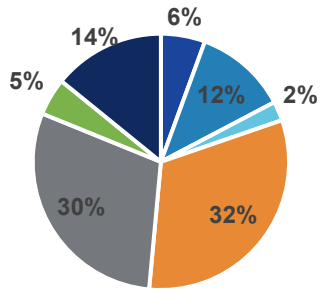
Acidovorax *Burkholderia* *Delftia*
Pseudomonas *Ralstonia* *Serratia*
Sphingomonas

Europe, n = 7691



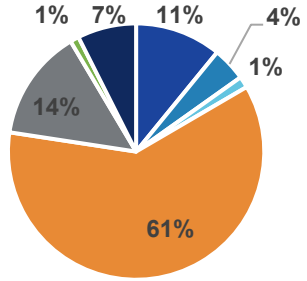
Acidovorax *Burkholderia* *Delftia*
Pseudomonas *Ralstonia* *Serratia*
Sphingomonas

North America, n = 24595



Acidovorax *Burkholderia* *Delftia*
Pseudomonas *Ralstonia* *Serratia*
Sphingomonas

South America, n = 633



Acidovorax *Burkholderia* *Delftia*
Pseudomonas *Ralstonia* *Serratia*
Sphingomonas

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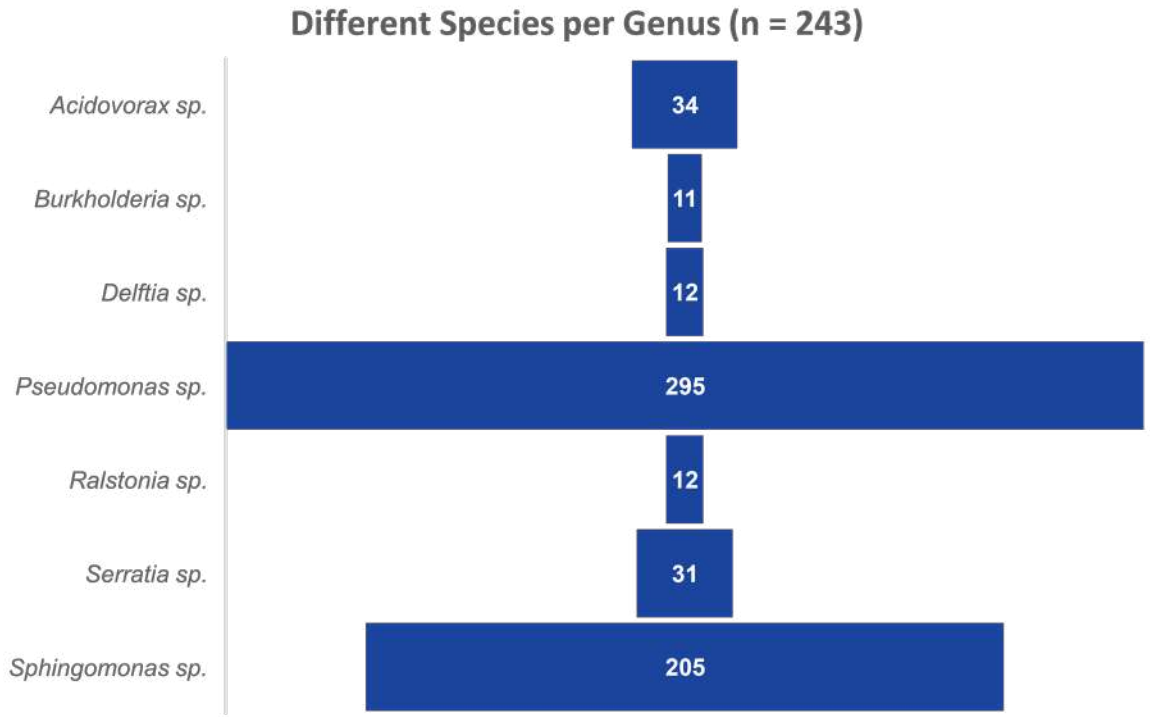
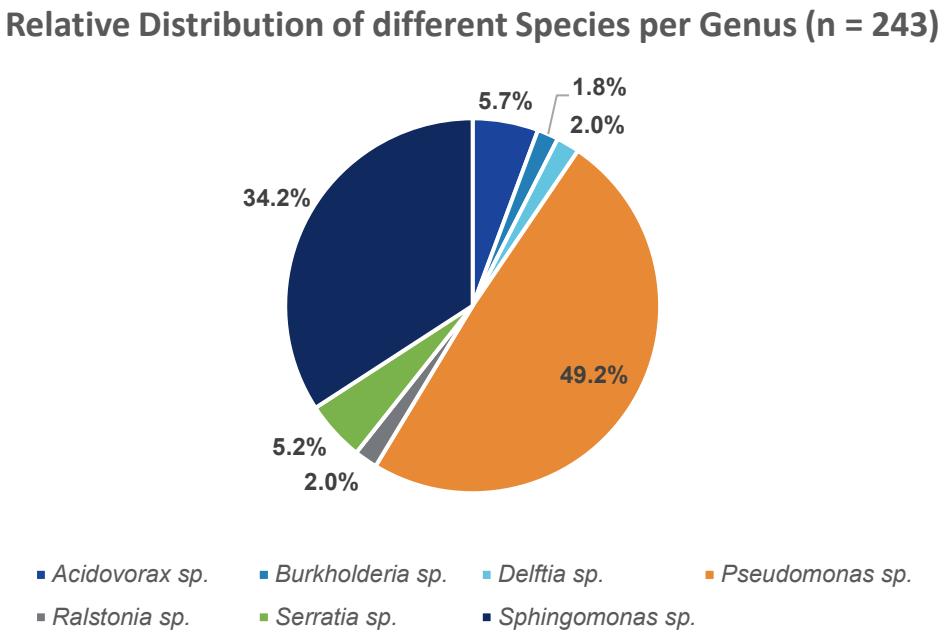
Number of different Species per most commonly isolated Biofilm Genera (n = 35664)





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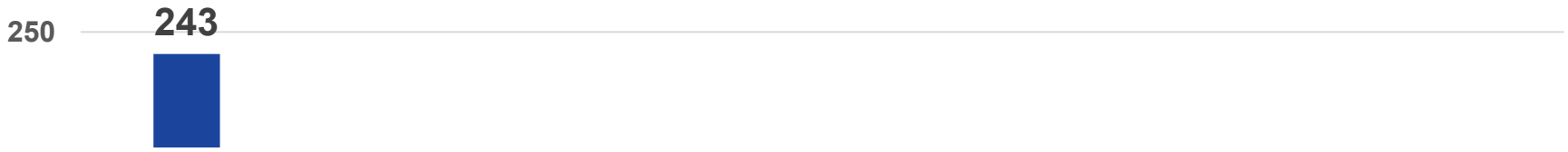
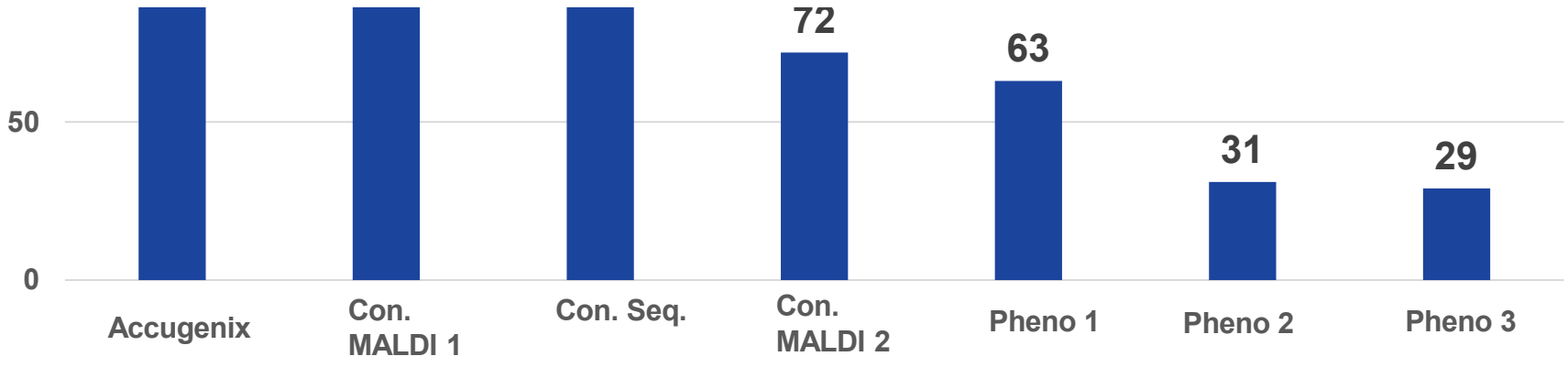
Species Entries per most commonly isolated Biofilm Genera in different ID System Libraries



Taxa found in Dialysis Water

Gram - Staining	Number
Gram – negative Taxa	28
Gram – positive Taxa	10

Number of different Species within these taxa in total (Accugenix – Charles River): 1215



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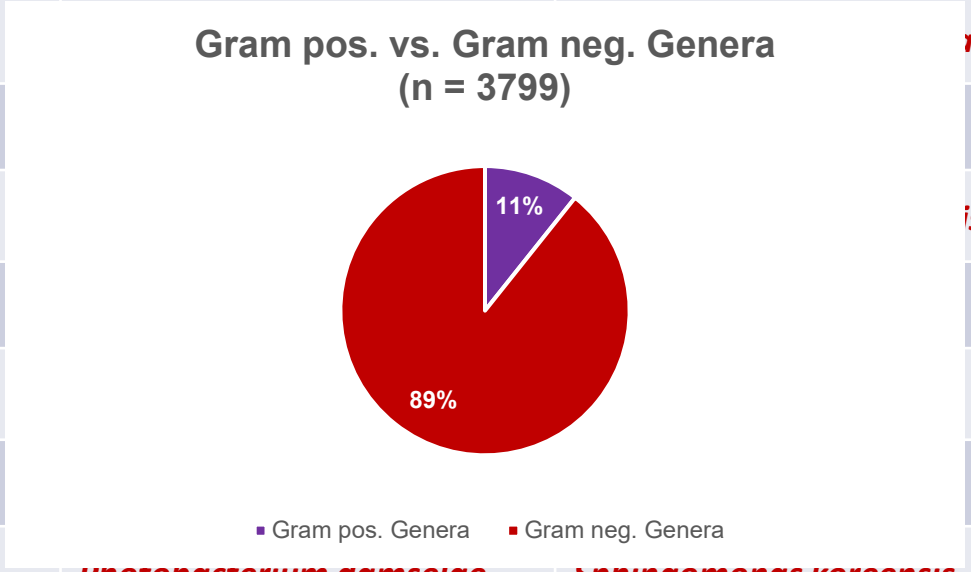
Case study – Organisms linked to Water Systems

Gram positive / **Gram negative** Species mentioned in the below publications evaluating water organisms of concern

Acidovorax delafieldii	*Carnobacterium maltaromaticum*	*Leifsonia aquatica*	*Pseudomonas fluorescens*	*Sphingomonas (Novosphingobium) stygia*
Acidovorax temperans	*Caulobacter henricii*	*Methylobacterium aquaticum*	*Pseudomonas monteilii*	*Sphingomonas (Sphingopyxis) terrae*
Aeromonas hydrophila	*Chryseobacterium indologenes*			*Stenotrophomonas maltophilia*
Aeromonas punctata	*Citrobacter freundii*			*Streptococcus sanguinis*
Aeromonas salmonicida	*Comamonas testosteroni*			*Tenacibaculum maritimum*
Aeromonas veronii	*Cupriavidus necator*			*Variovorax paradoxus*
Aquabacterium parvum	*Delftia acidovorans*			*Vibrio fluvialis*
Bradyrhizobium elkanii	*Edwardsiella ictaluri*			*Vibrio harveyi*
Bradyrhizobium japonicum	*Edwardsiella tarda*	*Photobacterium damselae*	*Sphingomonas koreensis*	*Vibrio parahaemolyticus*
Brevundimonas vesicularis	*Flavobacterium aquatile*	*Phyllobacterium myrsinacearum*	*Sphingomonas (Blastomonas) ursincola*	*Vibrio vulnificus*
Burkholderia cepacia complex	*Flavobacterium columnare*	*Pseudomonas alcaliphila*	*Sphingomonas paucimobilus*	*Yersinia ruckeri*



References:

1. Popovic et al. Environmental Research 152 (2017) 7–16. Differentiation of environmental aquatic bacterial isolates by MALDI-TOF MS.
2. Sandle T. SOJ Microbiol. Infect. Dis (2015) 3(2): 1-8. Characterizing the Microbiota of a Pharmaceutical Water System - A Metadata Study.
3. Kulakov et al. Applied and Environmental Microbiology (2002) 68(4) 1548–1555. Analysis of Bacteria Contaminating Ultrapure Water in Industrial Systems.
4. Martino et al. Rev Latinoam Microbiol. 1998 Jul-Dec;40(3-4):142-50. Identification of bacteria in water for pharmaceutical use.

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Case study – Organisms linked to Water Systems

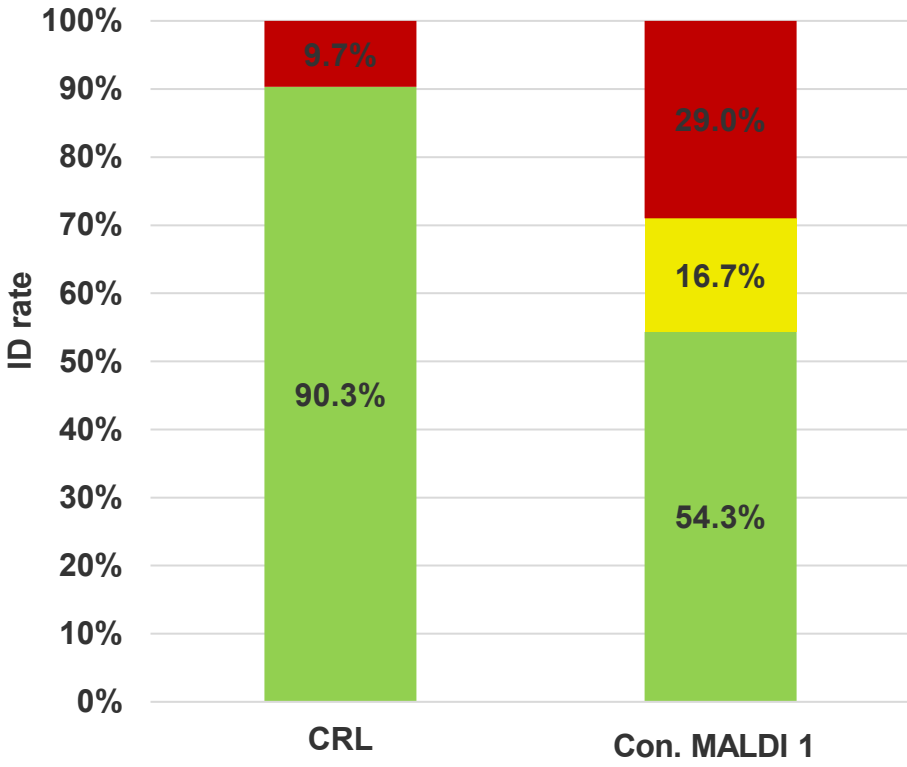
A subset of **Gram positive** / **Gram negative** species recovered to date from a global water study being conducted by CRL in collaboration with our customers.

	Burkholderia cepacia complex	*Pedobacter nutrimenti*	new
new	*Caulobacter sp.*	*Ralstonia insidiosa*	new
new	*Cupriavidus malaysiensis*	*Ralstonia pickettii*	
new	*Mesorhizobium australicum / erdmanii / japonicum*	*Rhizobium jaguaris / lusitanum / rhizogenes*	new
	Methylobacterium fujisawaense / oryzae / phyllosphaerae	*Rhizobium tropici*	new
new	*Methylobacterium longum / phyllostachyos*	*Sphingomonas aquatilis / melonis*	new
new	*Methylobacterium radiotolerans*	*Sphingomonas echinoides*	new
new	*Mucilaginibacter sp.*	*Sphingomonas sp.*	new
new	*Mycobacterium fortuitum*	*Sphingomonas(Sphingobium) yanoikuyae*	new
new	*Mycobacterium llatzerense*	*Undibacterium oligocarboniphilum*	new
new	*Paraburkholderia sp.*		

Common Challenges of Identifying Water Isolates

- "Water germs" are most of the time highly challenging isolates for pharmaceutical manufacturers. Thus, detailed knowledge of the water microflora can become a decisive need for evaluating the risk of a pharmaceutical production and must be based on an accurate identification.
- Existing culture methods (R2A-, /R3A – agars are minimal media) relinquish only a small part of the existing microflora, an even smaller part when dealing with biofilms.
- Water samples require often additional subculturing which is challenging due microorganisms being in „survival" rather than fully active mode due the actual life conditions of pharmaceutical water systems, but phenotypic and, to a lesser extent also MALDI systems, require fresh and metabolically intact isolates.
- A species entry for MALDI is based on strain representation which is composed of various spectra. Especially for water isolates, the strain and as such spectravariation can be complex. This variation can result in a significantly lower identification rate than the actual species presence in a library would suggest.
- **The outcome: The real identification rate of water isolates for not on sequencing based ID systems is usually lower than the number of library entries is reflecting. Considering the physical fitness of water isolates and the need for accuracy, achieving a successful accurate identification with a not by sequencing backed up ID solution is even more questionable.**

Impact of CRL Library and optimized threshold



MALDI SCORE KEY	CRL	Con. MALDI 1
Species ID	≥1.75	≥2.0
Low confidence ID	NA	≥1.7 and <2.0
No ID	<1.75	<1.7

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VBNC Pathogens Commonly isolated from Water in Yellow

microrite
achieving success through expertise

Pathogens known to enter the VBNC state

Aeromonas hydrophila
Helicobacter pylori
Serratia marcescens
A.salmonicida
B.Klebsiella aerogenes
C.Shigella dysenteriae
Agrobacterium tumefaciens
K. pneumoniae
S. flexneri
Burkholderia cepacia
K. planticola
S. sonnei
B. pseudomallei
Legionella pneumophila
Streptococcus faecalis
Campylobacter coli
Listeria monocytogenes
Vibrio alginolyticus
C. jejuni
Mycobacterium tuberculosis
V. anguillarum
Francisella tularensis
S. typhimurium

C. lari
M. smegmatis
V. campbellii
Cytophaga allerginae
Pasteurella piscicida
V. cholerae
Enterobacter aerogenes
Pseudomonas aeruginosa
V. harveyi
E. cloacae
P. syringae V. mimicus
Enterococcus faecalis
Ralstonia solanacearum
V. parahaemolyticus
E. hirae
Rhizobium leguminosarum
V. shiloi
E. faecium
R. meliloti
V. vulnificus (types 1 & 2)
Erwinia amylovora
Salmonella enterica
Xanthomonas campestris
Escherichia coli (including EHEC)
S. typhi
X. axonopodis pv. citri

How to identify these pathogens from water systems, when common methods are relying on pure or metabolically intact cultures?

Yes, with sequencing. But common 16S rDNA sequencing requires a *pure* culture.

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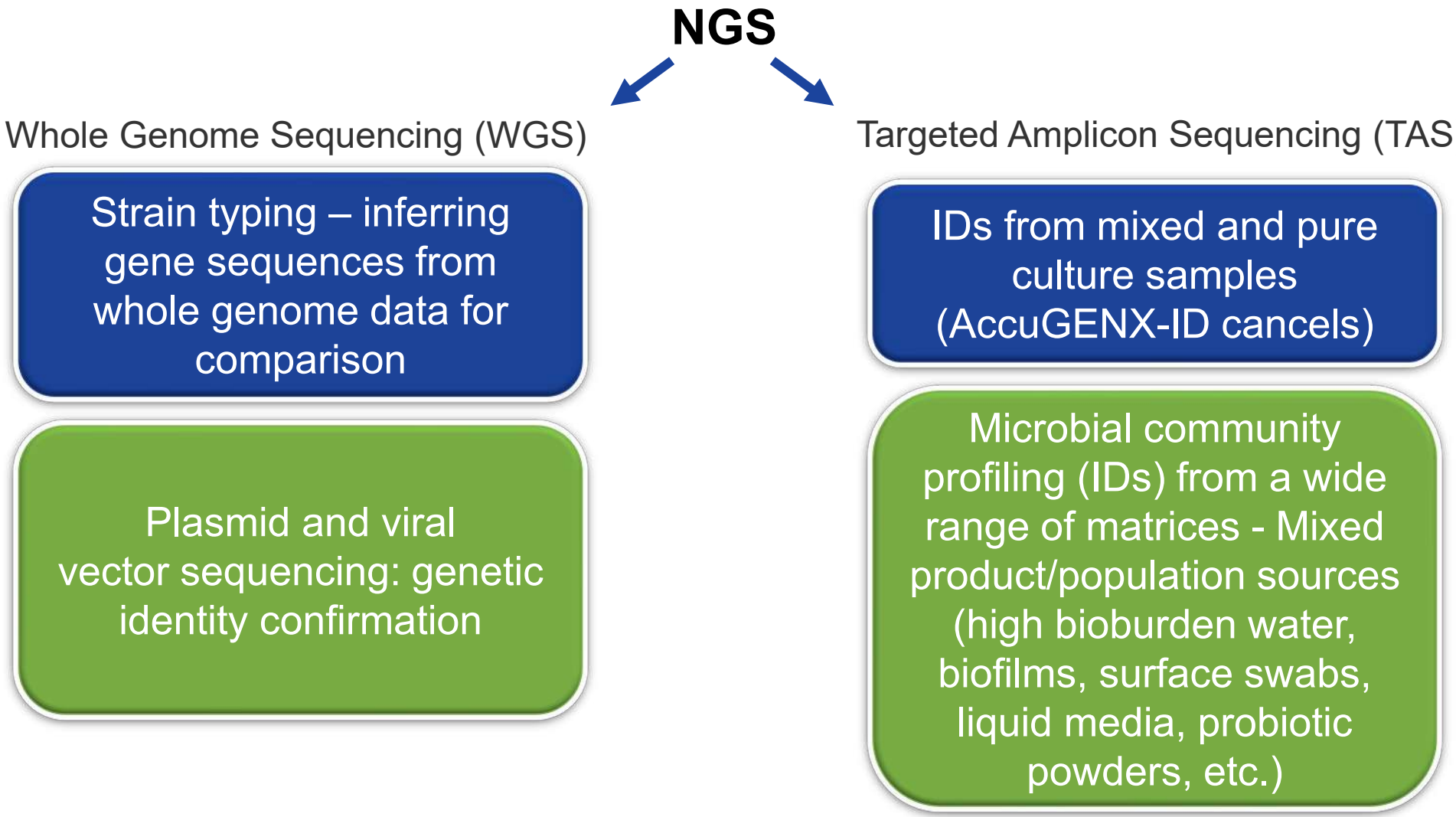
(Mixed) VBNCs are Ideal Candidates for Next Generation Sequencing (NGS)

- NGS can help for **mixed colonies or difficult to cultivate samples** which Sanger cannot analyze,
- Sanger is limited to the target genes you decide to analyze. With NGS, you could sequence the whole gene and then run the bioinformatic pipeline to anaylze the sequences you want, potentially every sequence,
- If we consider Strain Typing, with Sanger we need to know in advance which genes we need to amplify in dependence of an accurate species level ID and then sequence, to define the primers to be used for Strain Typing. With NGS the whole genome (Whole Genome Sequencing, WGS) is sequenced, followed by the use of bioinformatics to look at the sequences to be compared.

(Mixed) VBNCs are Ideal Candidates for Next Generation Sequencing (NGS)

Two workflows – four primary applications



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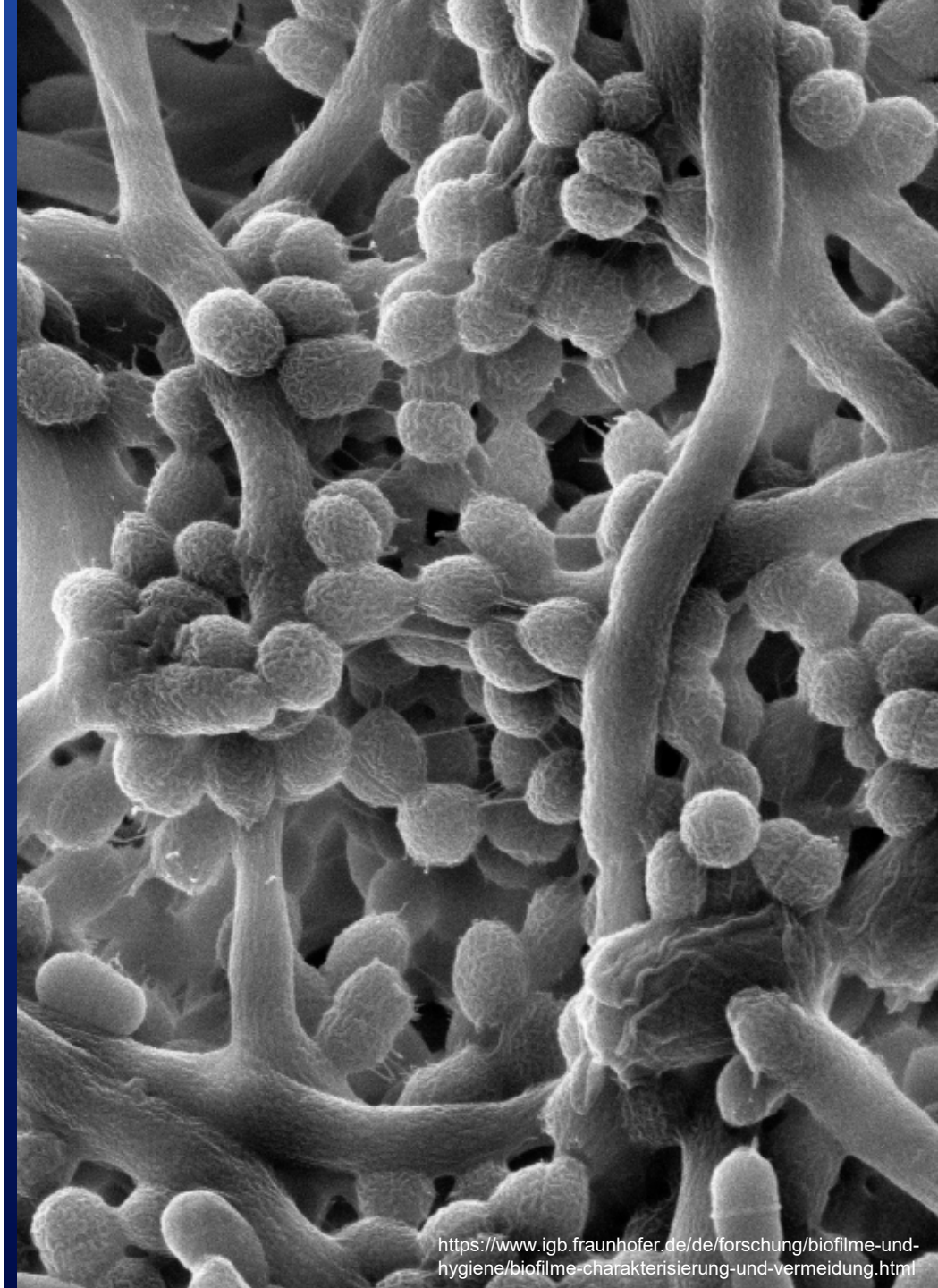
The costly usual „Treatment“ of a Biofilm – a Real World Example



https://www.igb.fraunhofer.de/de/forschung/biofilme-und-hygiene/biofilme-charakterisierung-und-vermeidung.html

Treating a Biofilm with a Focus on Cost Savings is very expensive.

Sept. 2021:

- Customer realized significant bioburden increase in finished liquid product (batch had to be discarded),
- Final product bottle tested positive on Celsis meaning the presence of microorganisms via ATP was proved,
- Inoculum, taken from the final product bottle, subcultured on TSA, SDA, CA & Mac Conkey agar resulted in bacterial growth on TSA and McConkey,
- Although colony morpholgy was „highly similar“, the phenotypic system unreliably identified some of these samples,
- Sample was submitted to AGX for AccuPRO – ID (MALDI backed up by sequencing“ service and got identified as *Serratia marcescens,*
- Due to the criticality of the sample origin and the species described as biofilm member, AGX recommended to take additional samples from the water system and from at least one intermediate product.

Oct. 2021:

- Two water samples from the „recirculatoin tour“ and one intermediate product sample got isolated on TSA/McConkey. Phenotypic identification failed.
    - TSA plate A showed three morphologically different organisms,
    - McConkey plate shows two morphologically different organisms,
    - TSA plate B fully covered with morphologically „highly similar colonies“.
- AGX identified these samples with AccuPRO – ID as members of:
    - *Pseudomonas fluorescens group* - gram negative,
    - *Bacillus clausii* - gram positive,
    - *Ralstonia pickettii* - gram negative,
    - *Serratia marcescens* - gram negative.
- AGX postulated due to the microflora composition the presence of a biofilm and recommended to test further samples from „corners“, valves and intermediate products.
- Customer declined the recommendation and cleaned „affected parts“ of the water system with heat. Afterwards, count was again within specs, no morphologically different organisms have been isolated.

Treating a Biofilm with a Focus on Cost Savings is very expensive.

Early February 2022:

- Counts out of specs again, sample again isolated from finished liquid product (batch had to be discarded),
- Isolate was again identified as *S. marcescens* by AGX,
- AGX recommended again to collect additional samples from the water system and intermediate products.

Mid of February 2022:

- Five samples, subcultured on TSA, McConkey, have been submitted to AGX for an identification,
- Two samples from „Warm tour“ have been identified as *Ralstonia pickettii* (Gram negative),
- One sample from an intermediate product was identified as *Serratia marcescens / nematodiphila / ureilytica* (Gram - negative), although submitted as “morphologically highly similar”,
- One sample from “Pre – filtering” was identified as *Pseudomonas fluorescens group* (Gram - negative),
- One sample from an “elbow shaped pipework connection/valve” was identified as *Delftia acidovorans / lacustris* (Gram - negative), another one as *Acidovorax delafieldii* (Gram - negative),
- ID data confirmed the postulation of AGX from the previous year and proved the presence of a biofilm, spreading across the water system resulting in contaminating the final product already at intermediate stages,
- AGX recommended to improve water monitoring by adding sampling points to critical areas due to the emergence of new species, e. g. *Acidovorax delafieldii*, *Delftia acidovorans / lacustris*. Moreover, strain typing was suggested for isolates that belonged to the same species but are from various sampling points in order to identify the origin of the contamination in the final product. This would help to exclude other potential contamination sources and allow for a specified, targeted solution to get rid off the contamination origin,
- Customer declined recommendation and cleaned „affected parts“ of the water system with heat/chemical treatment.

Treating a Biofilm with a Focus on Cost Savings is very expensive.

September 2022:

- Counts have been out of specs again, final product was contaminated again, batch had to be discarded again.
- Sample was again identified by AGX as *Serratia marcescens* (final product), another one as *Ralstonia pickettii* (intermediate product).
- Further, additional samples from the water system have been identified by AGX as members of *Acidovorax sp*., of *Delftia sp*. and of *Pseudomonas sp.*
- Customer cleaned again „affected parts“ of the water system with heat/chemical treatment.

Outcome so far:

- Due to AGX results and recommendations, customer stopped using a phenotypic system for identification, outsources to AGX instead and might further use a CRL portable endotoxin measurement system for detecting endotoxins as an early indicator of Gram negatives.
- Discussion with Sr. Mgmt. about increased water monitoring activities and identification of the contamination origin with the help of a broader sampling, identification and strain typing approach are ongoing. Once the contamination origin is identified, customer might re – design and replace certain parts of the water system, e. g. dead corners.

Painful lesson learned? Hopefully, because…

- Three times an entire final product batch was discarded,
- Three times the (almost) identical sanitization approach was followed without any sustainable, long – term effect,
- Three times an ineffective CAPA was initiated and executed, involving several stakeholders from production, QA, the lab and Sr. Management.

Treating a biofilm as an opportunity to save cost will only help to get rid of even more money, but not the biofilm.

Endotoxins in a Product? The dilemma with Water.

- The presence of endotoxins in an, e. g. aqueous, product is *not* necessarily linked to gram – negative organisms in the water system.
- A proper LAL investigation with a focus on the nature of the analyte is equally important, when dealing with microbial issues of a water system.

Contact Us

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08/17/2023

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Thank you!
Questions?

charles river

Speaker Introduction

charles river

Alex Tan

Senior Technical Services Specialist at Charles River Laboratories

Alex Tan is a Senior Technical Services Specialist with Charles River Laboratories Singapore, specializing in endotoxin testing in various platforms. In his role, Alex supports the Technical Services for customers in the APAC region, he also drives the onboarding of new customers and is responsible for the initial training and validation/application support for customers as well as conducting method development and feasibility testing for the Endosafe® brand of products. His last position with the company was Team Lead, LAL and Microbiology testing, where he was responsible for protocol development and delivery of subcontract LAL and bioburden services. Alex has a degree in Molecular Biology and Biotechnology.

Difficult to resolve LAL investigation

Alex Tan

Senior Technical Services Specialist

Charles River Microbial Solutions (Singapore)

PDA Parenteral Drug Association
Connecting People, Science and Regulation®

2023 PDA Pharmaceutical Manufacturing and Quality Conference

CONNECTING PEOPLE SCIENCE AND REGULATION®

Case Study 1

Case Study
Customer was using KCA reagent During product validation saw these results Questions they asked: ▪ What is going on here?

Dilution Factor	Endotoxin Units/mL	Spike Recovery
1 : 50	< 0.25	110%
1: 100	< 1	147%
1: 200	2.16	195%
1: 400	6.3	262%
1: 800	28.6	307%

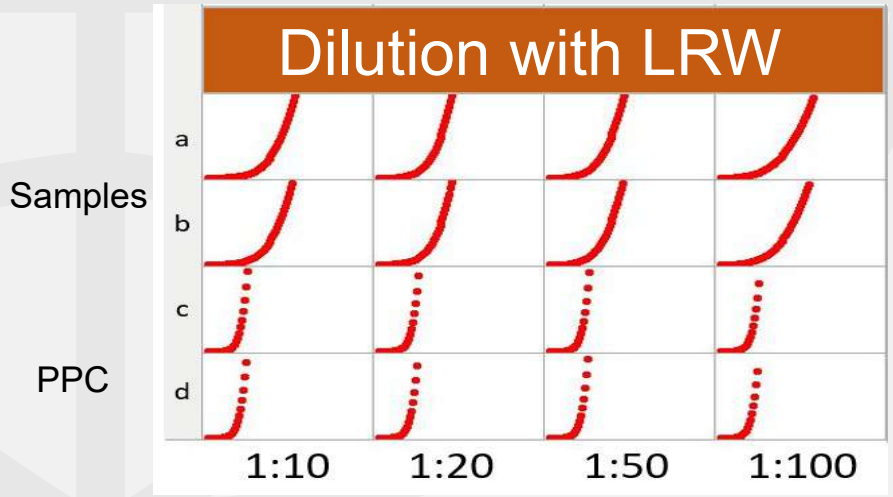
Case Study 1

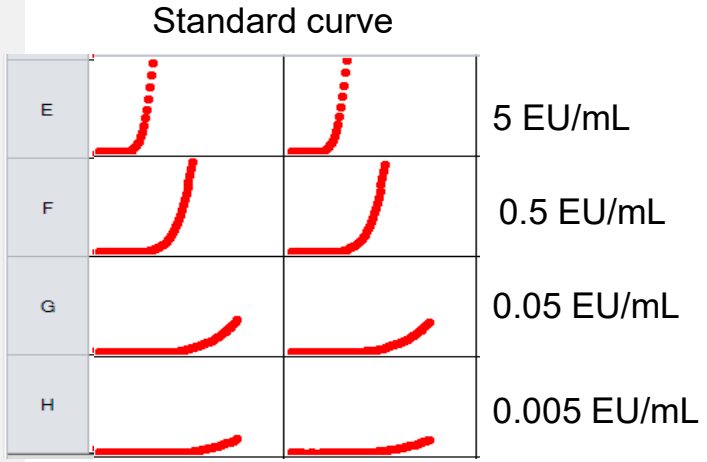
Customer was using KCA reagent

During product validation saw these results

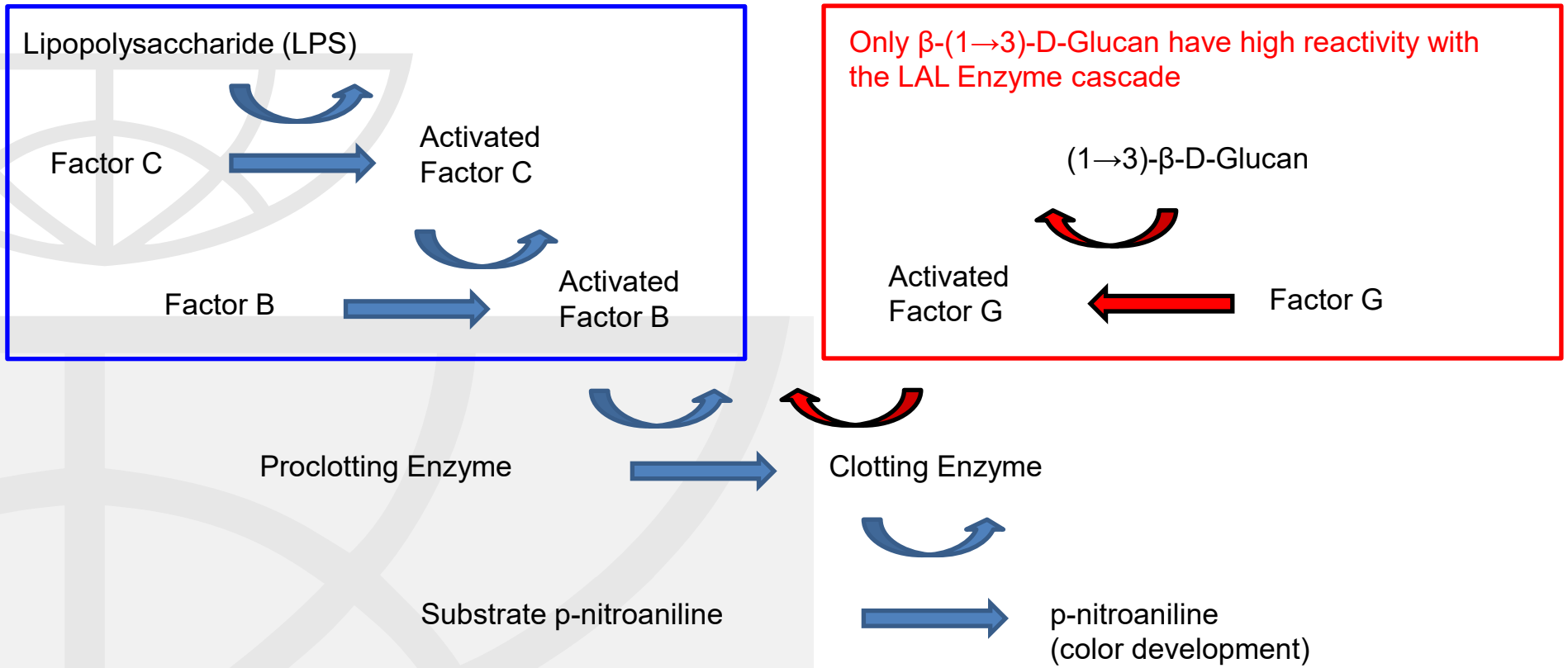
What is going on here?

Dilution	LAL Reagent Water	
	EU/mL	Spike Recovery
1:10	0.0269	144
1:20	0.0576	128
1:50	0.0639	135
1:100	0.0287	150

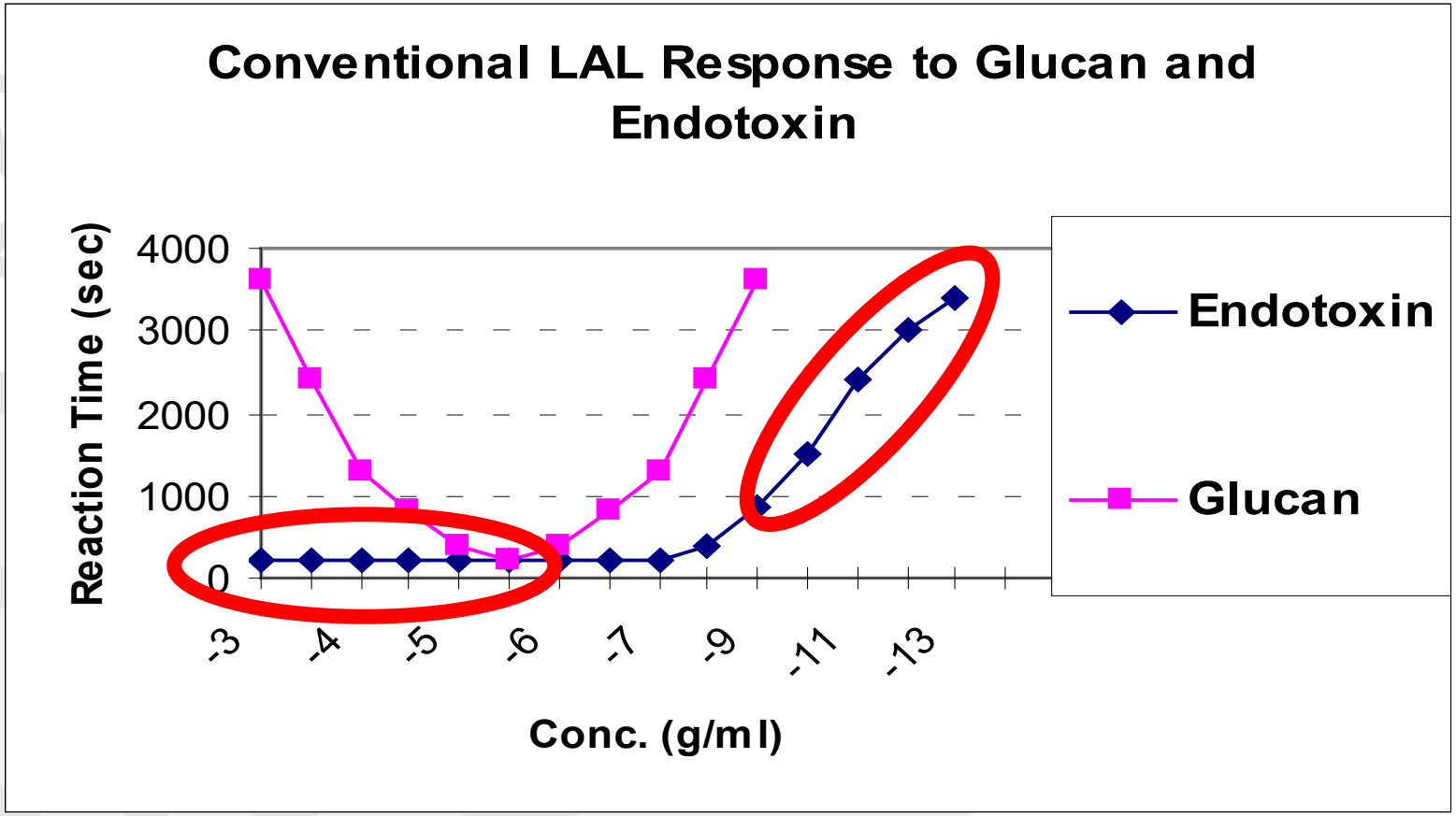




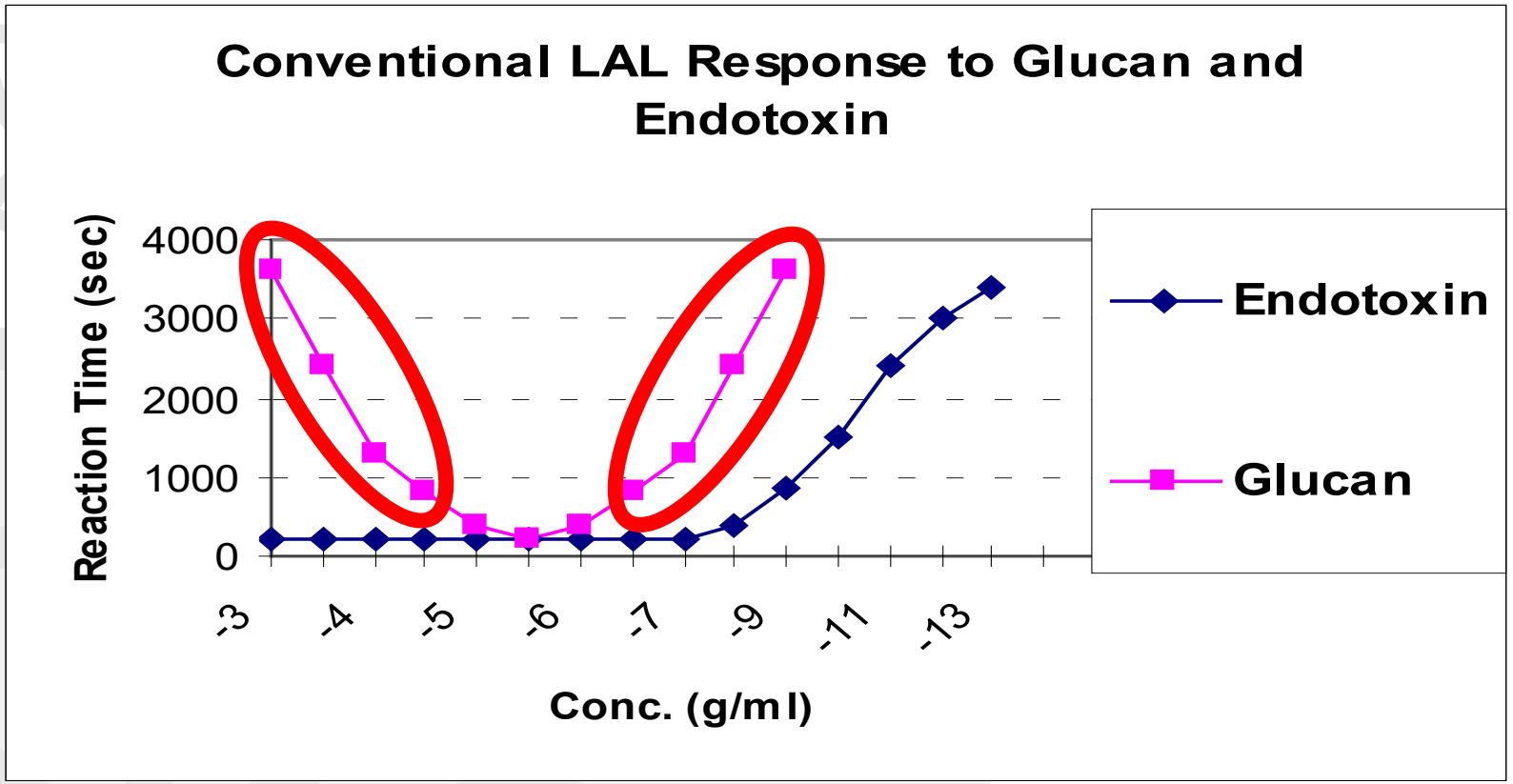
Case Study 1: Beta Glucans



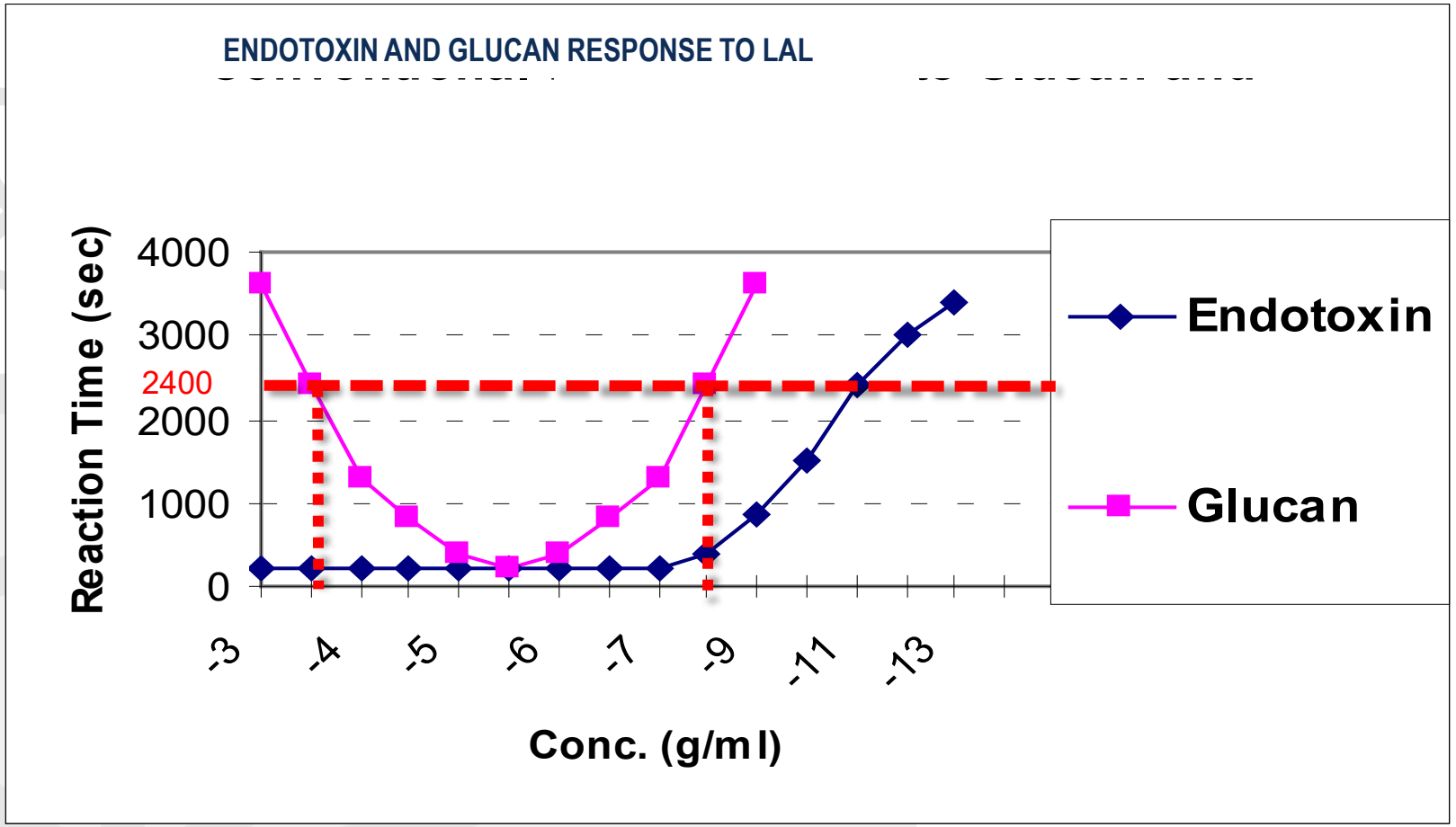
Case Study 1: Beta Glucans



Case Study 1: Beta Glucans



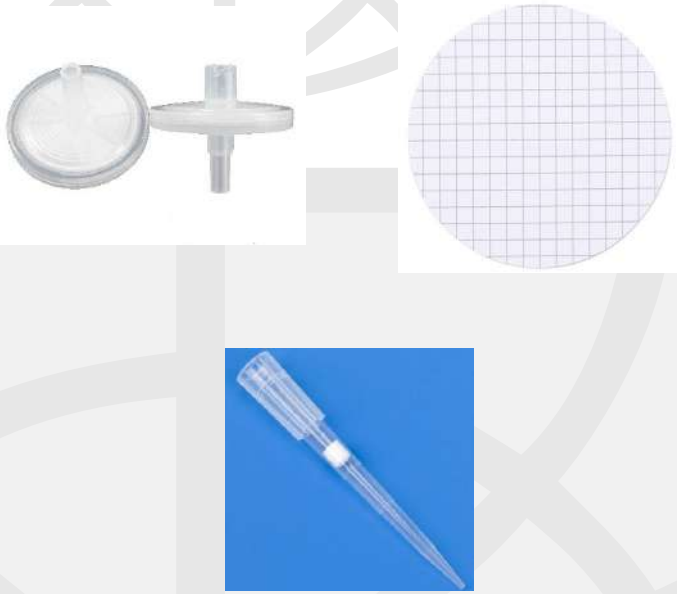
Case Study 1: Beta Glucans



Case Study 1: Beta Glucans

- Specific to β-(1→3)-D-glucan
- Response varies significantly depending on type of β-Glucan
- U-shaped dose-response curve
- Endotoxin and β-Glucans Can React Synergistically

Case Study 1: Common sources (Beta Glucans)

Cellulose Materials	Naturally-derived raw materials	Microbial Contaminations
	 	 

Case Study 1: Beta Glucans (Resolving the problem)

- Glucan Cartridges
  - Quantify (1,3)-β-D glucans
  - Rapid, in-process test for investigational purpose
    - ~ 30 mins to results
  - Sensitivity range: 10 – 1,000 pg/mL
  - Mimics kinetic chromogenic method by measuring color intensity

- Endotoxin specific Buffer (BG600)
  - Reconstitute LAL lysate
  - Cartridges: Dilute with BG600

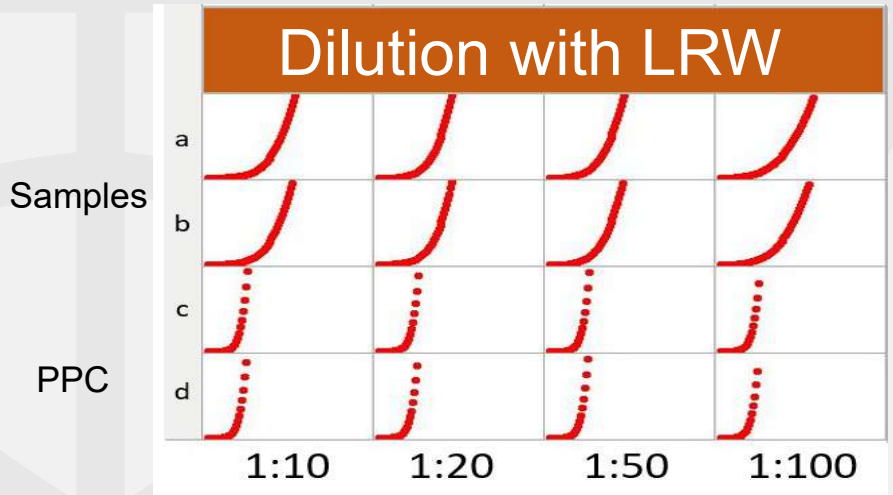
Case Study 1: Beta Glucan

Customer was using KCA reagent

During product validation saw these results

What is going on here?

Dilution	LAL Reagent Water	
	EU/mL	Spike Recovery
1:10	0.0269	144
1:20	0.0576	128
1:50	0.0639	135
1:100	0.0287	150

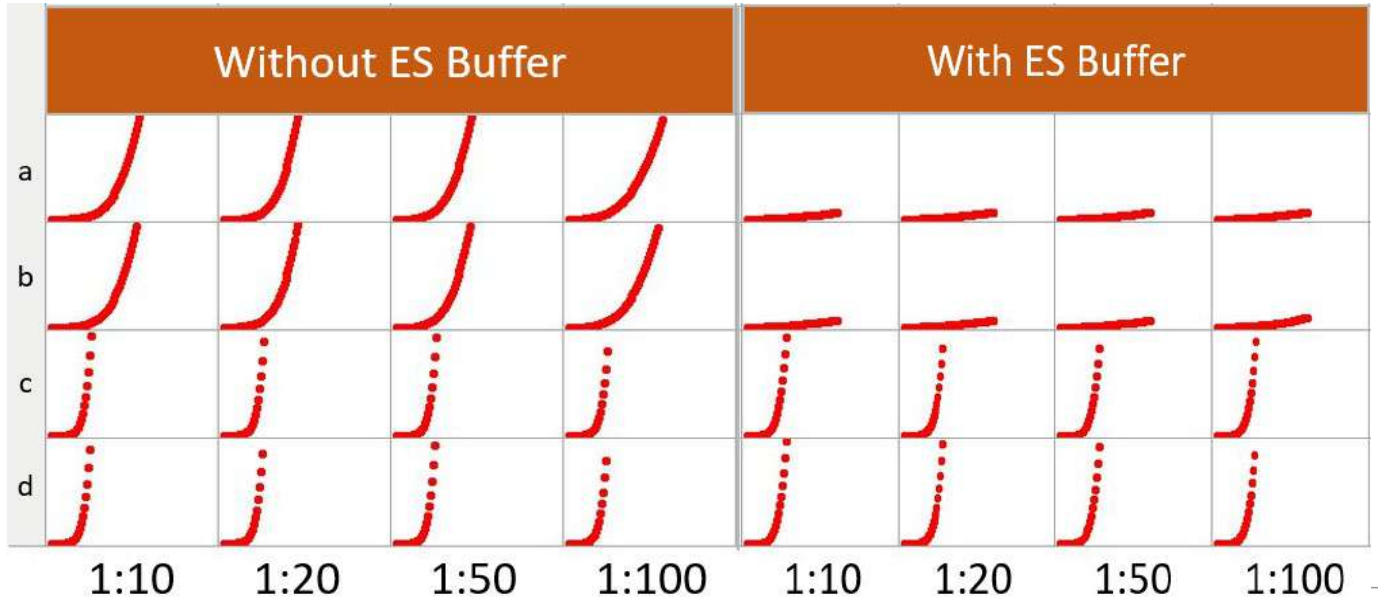


Case Study 1: Beta Glucans (Resolving the problem)

Dilution	Without ES Buffer		With ES Buffer	
	EU/mL	Spike Recovery	EU/mL	Spike Recovery
1:10	0.0269	144	<0.005	110
1:20	0.0576	128	<0.005	102
1:50	0.0639	135	<0.005	101
1:100	0.0287	150	<0.005	110

Sample

PPC



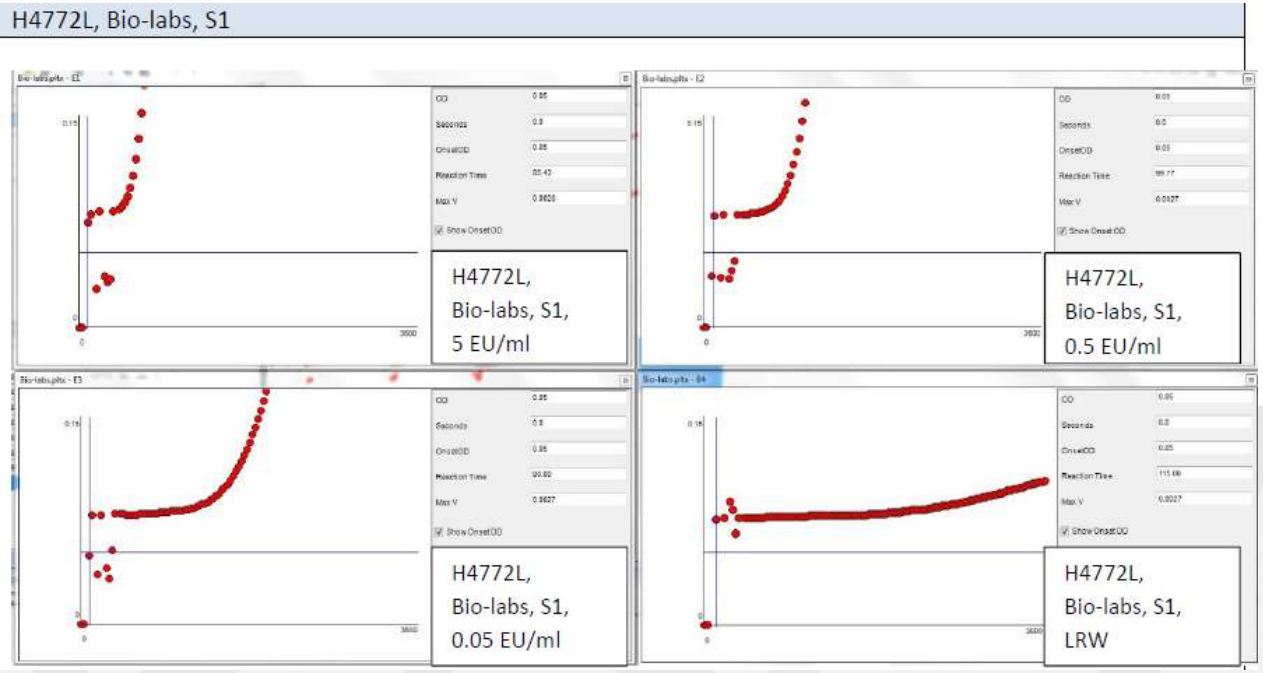
Case study 2

	Onset Times In Seconds					
EU/ml	Endosafe Data	Dec 2009	Mar 2010	Mar 2010	Apr 2010	Apr 2010
5.0	**591**	556	534	410	322	328
0.5	**981**	909	781	592	429	434
0.05	**1774**	1475	1129	880	593	571
0.005	**2700**	1811	1292	1561	984	888

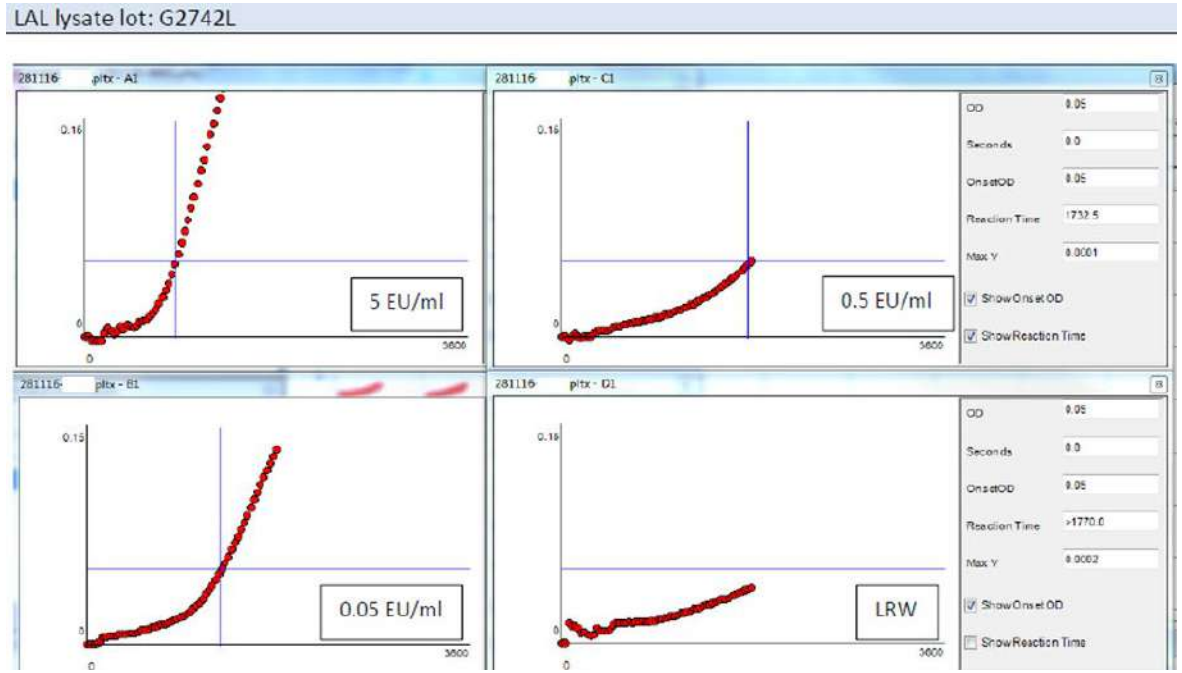
Plastic Accessories Were the Cause of the Fast Reaction Times:

- Plastic Pipettes
- Plastic Multi-Channel Reservoirs

Case Study 2: Plastic accessories(Pipette Tips)



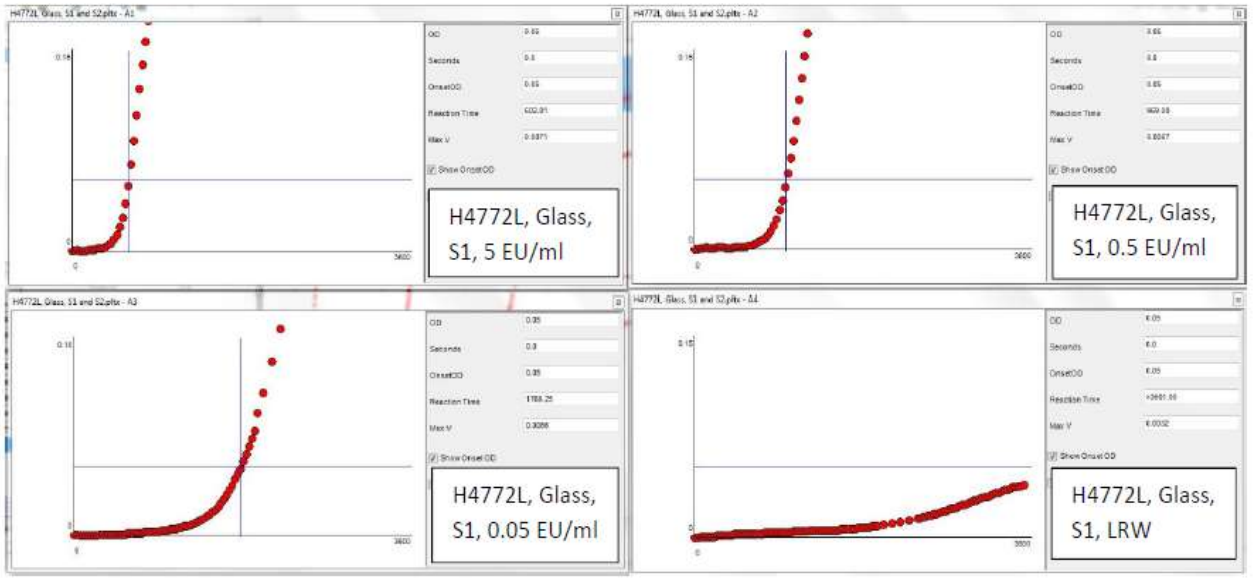
Plastic Pipette Brand A



Plastic Pipette Brand B

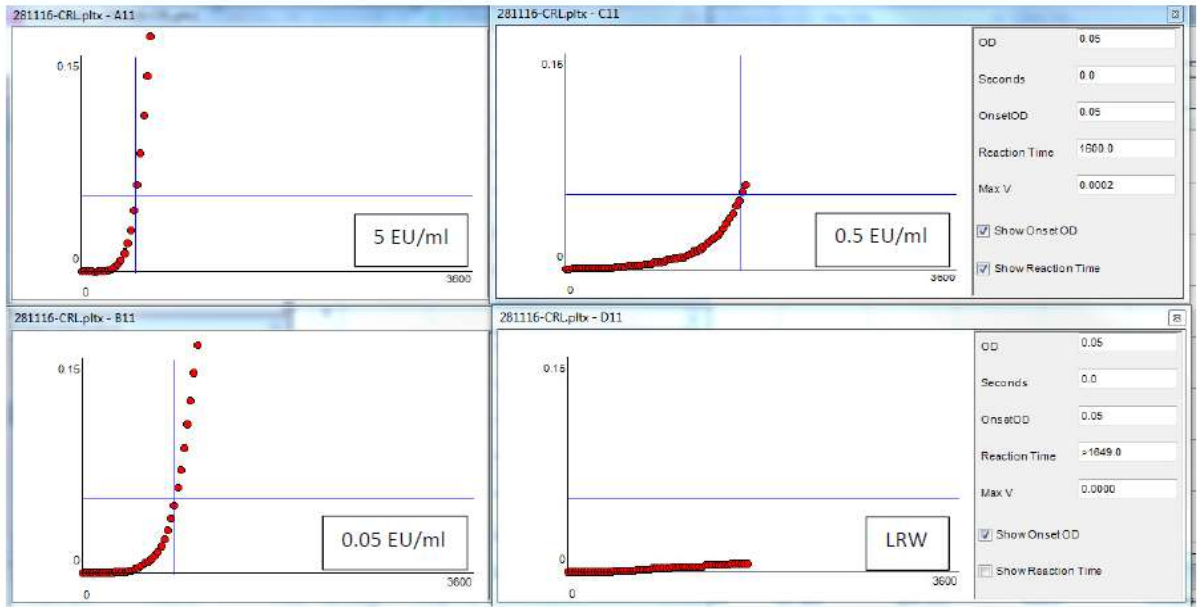
Case Study 2: Plastic accessories (Glass and Plastic Tips)

H4772L, Glass, S1



Glass pipettes

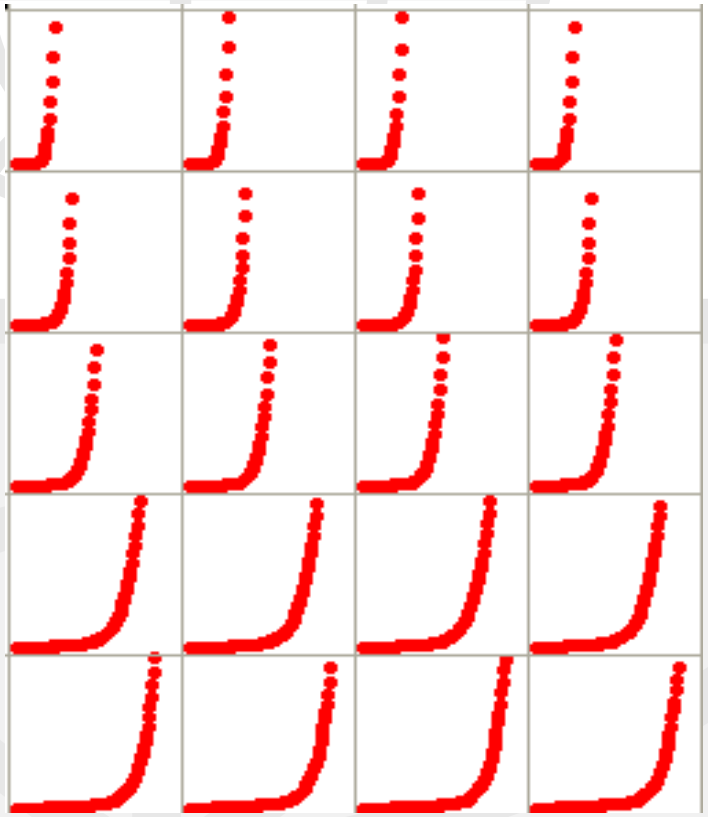
LAL lysate lot: G2742L



Not all plastics are bad

Case Study 2 Plastic accessories (Microtiter plates)

STANDARD CORONA DISCHARGE PLATE



5.0

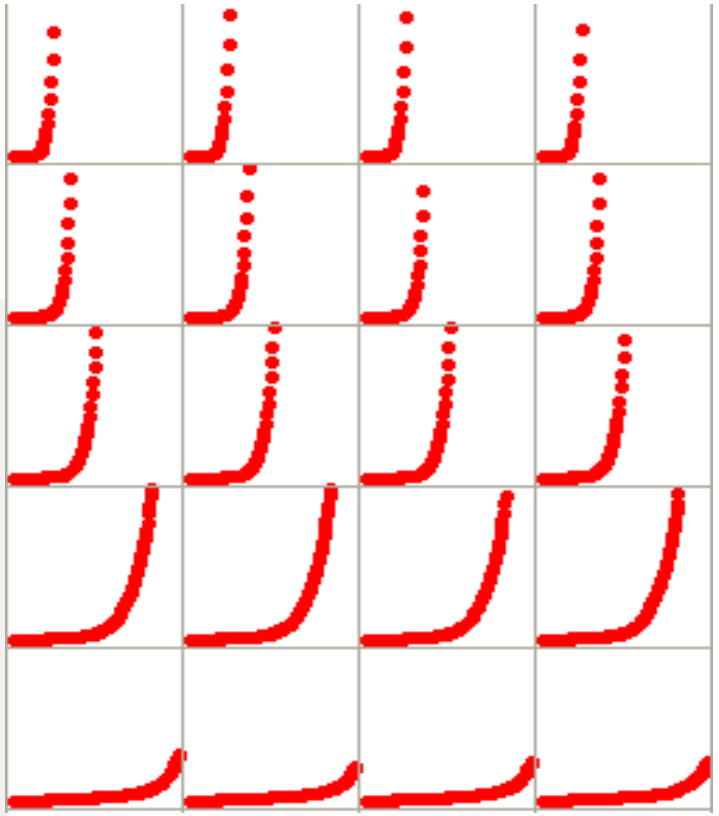
0.5

0.05

0.005

NWC

STANDARD VACUUM PLASMA PLATE



Plastic accessories: Microtiter plates

- **Tissue Culture Treatments**
- Modifies the surface properties without affecting bulk material
- Tissue Culture Processes are engineered to Saturate exposed surfaces
- Effects:
    - Imparts a net “negative” surface charge
    - Increases “wettability” of surface
    - Activates LAL

Tissue Culture Processes

- Corona Discharge:
    - Creates an atmospheric gas plasma
- Vacuum Plasma
    - Vacuum created
    - Pure O2 is generated
    - O2 plasma generated

Case study 3: Standard Curve Variabilities

- Standard Curve Validity criteria
  - Absolute correlation coefficient, **r**, must be greater than or equal to 0.980 for the range of endotoxin concentrations set up
  - Concentrations in your blank/Negative water control should be non-reactive
  - CV%

Is R value sufficient for your standard curve?

Case Study 3: Standard curve Variabilities

The accuracy of the test result depends on the accuracy of the standard curve

Why |R| value (linearity) is not enough to assess standard curve quality:

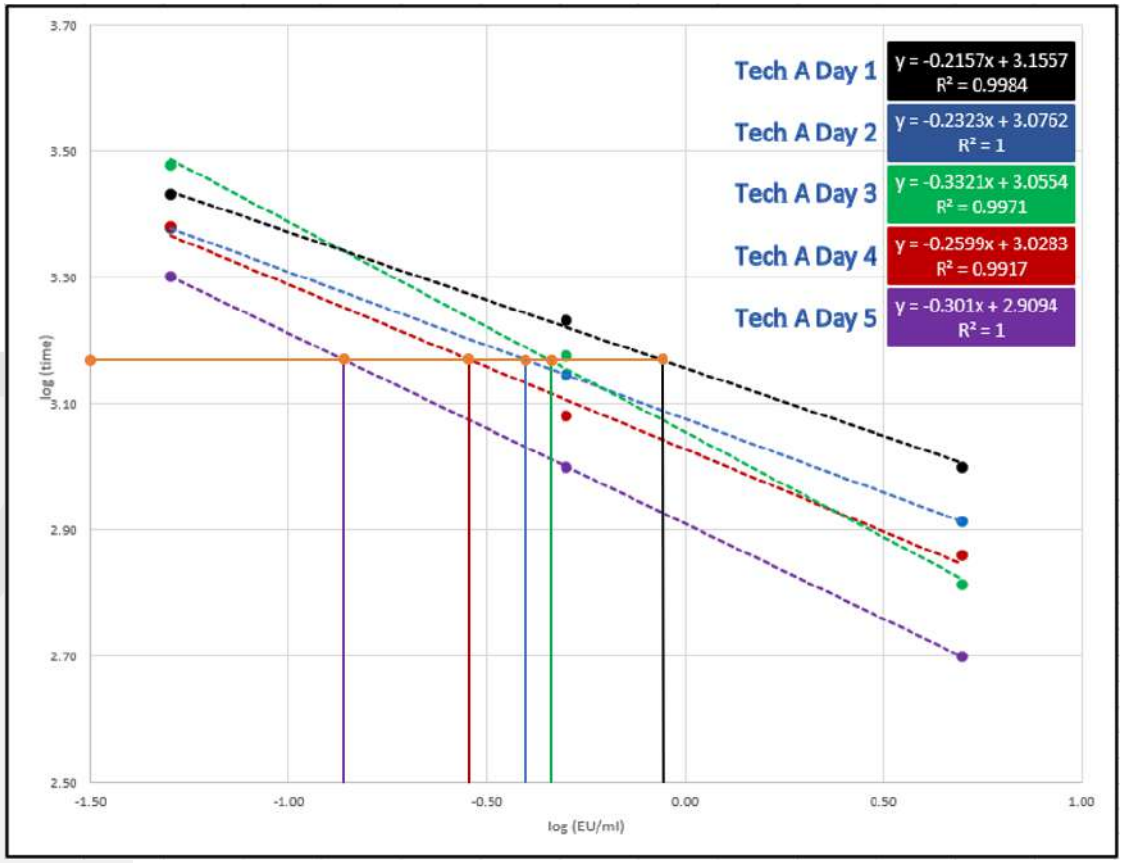
- Each user prepared curve will be different
- As the curves change, so do the sample endotoxin values

Example:

5 curves with |R| values between 0.9917 and 1.0

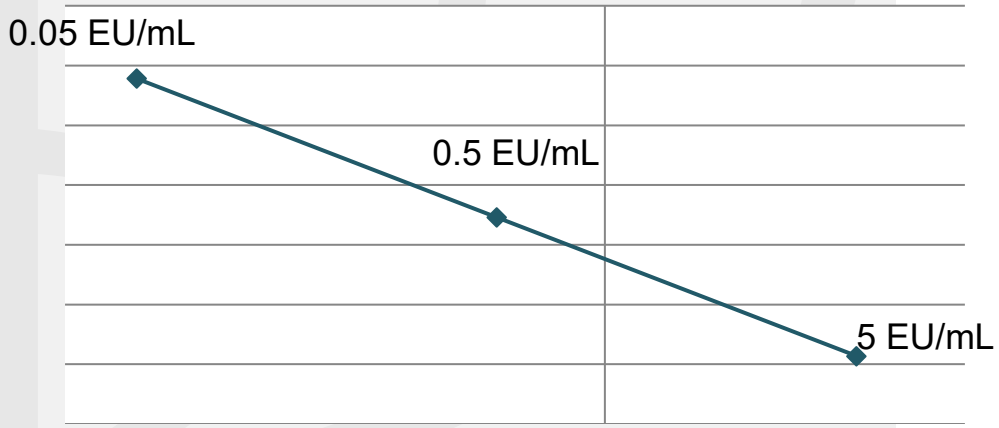
- same analyst
- same LAL lot
- same CSE vial
- same curve range
- same procedure

Gives 5 very different sample EU values for the same sample reaction time



Dissecting the Standard Curve

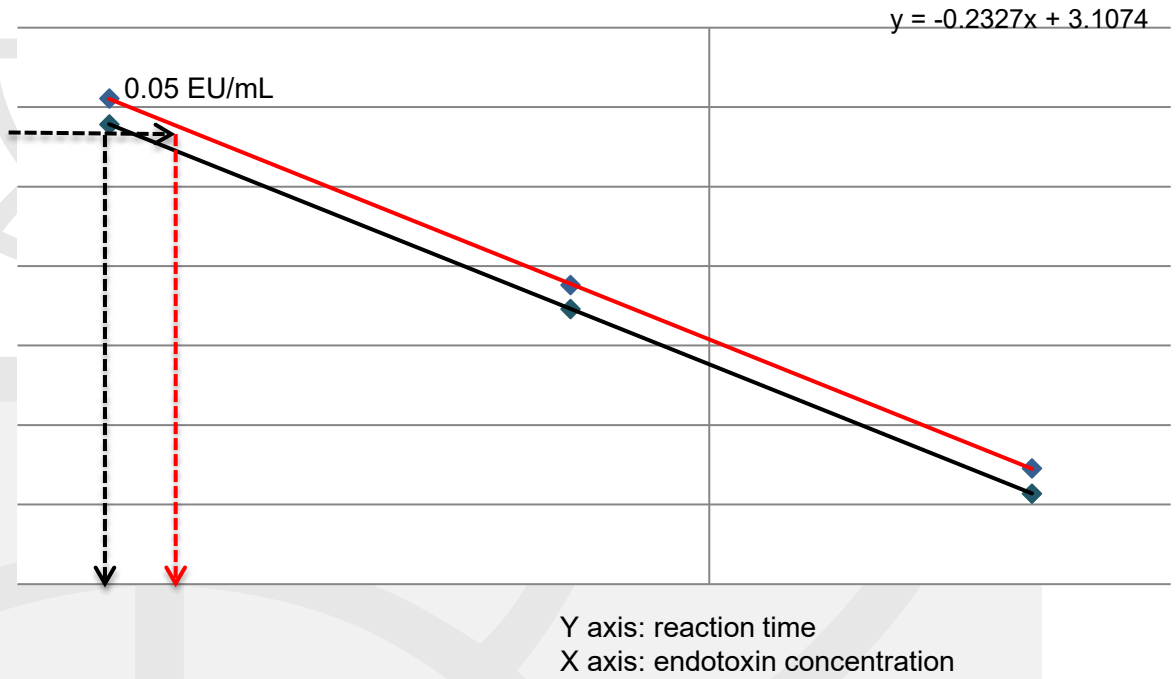
- Create a “benchmark” standard curve in Excel
  - |r| = 1.0
  - Slope = -0.2323
  - Y-intercept = 3.0762
- Study the curve by changing one attribute at a time.



EU/mL	Onset (seconds)
5.0	820
0.5	1400
0.05	2390

Dissecting the Standard Curve: Y-intercept

Change: Y-intercept + 1%



Benchmark curve:

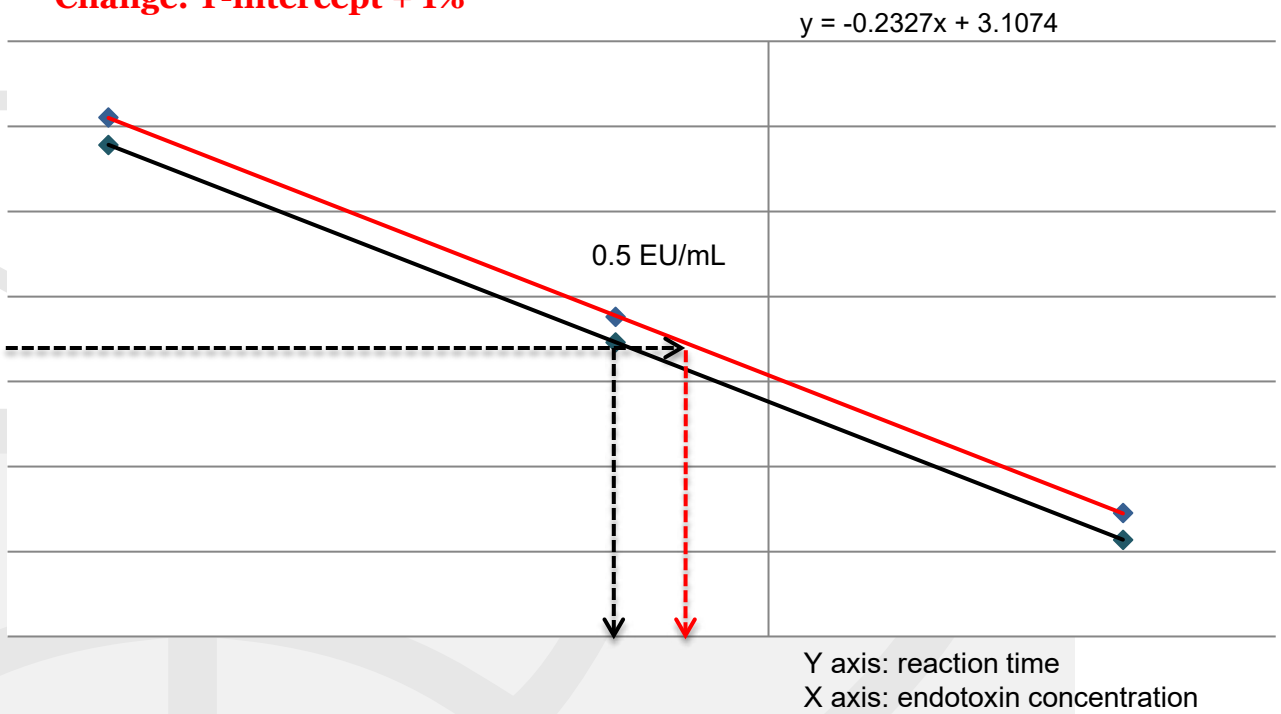
EU/mL	Onset (seconds)
5.0	882
0.5	1500
0.05	2575

2575 seconds reaction time for an unknown = 0.05 EU/mL on the benchmark curve (black)
2390 seconds reaction time for an unknown = 0.068 EU/mL on the modified curve (red)

Difference = 136% of nominal value

Dissecting the Standard Curve: Y-intercept

Change: Y-intercept + 1%



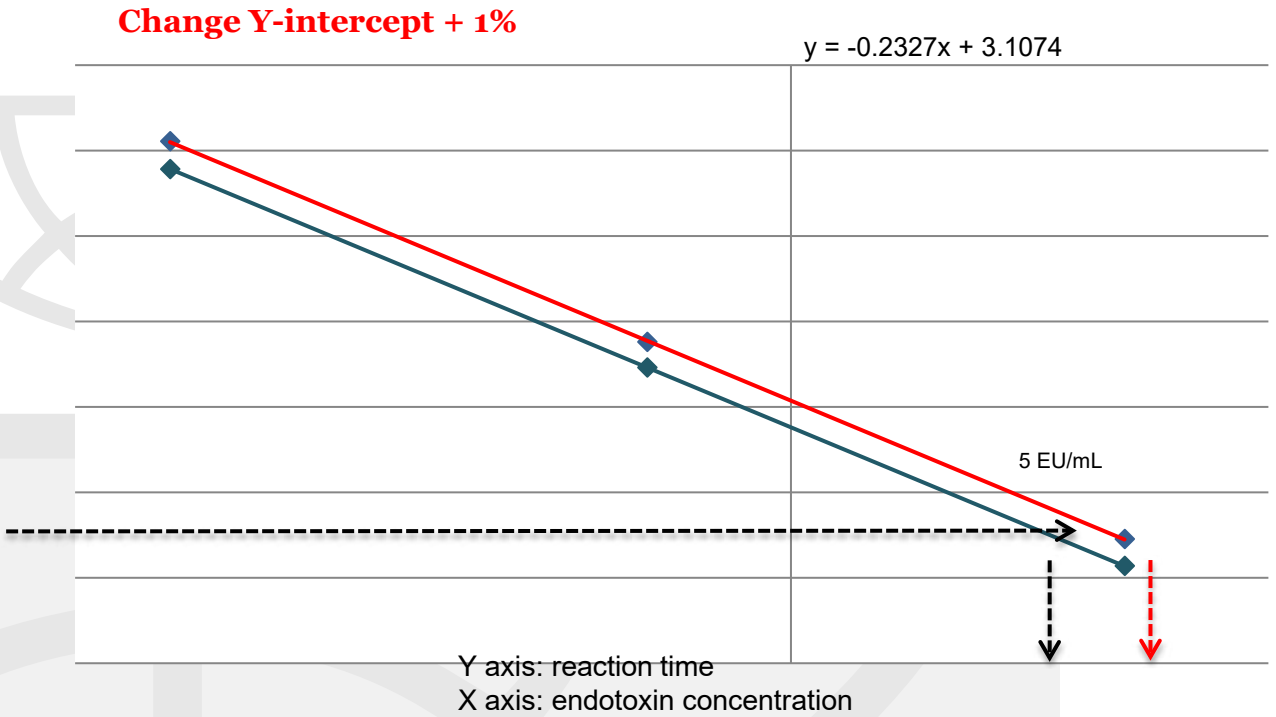
Benchmark curve:

EU/mL	Onset (seconds)
5.0	882
0.5	1500
0.05	2575

1500 seconds reaction time for an unknown = 0.5 EU/mL on the benchmark curve (black)
1400 seconds reaction time for an unknown = 0.68 EU/mL on the modified curve (red)

Difference = 136% of nominal value

Dissecting the Standard Curve: Y-intercept



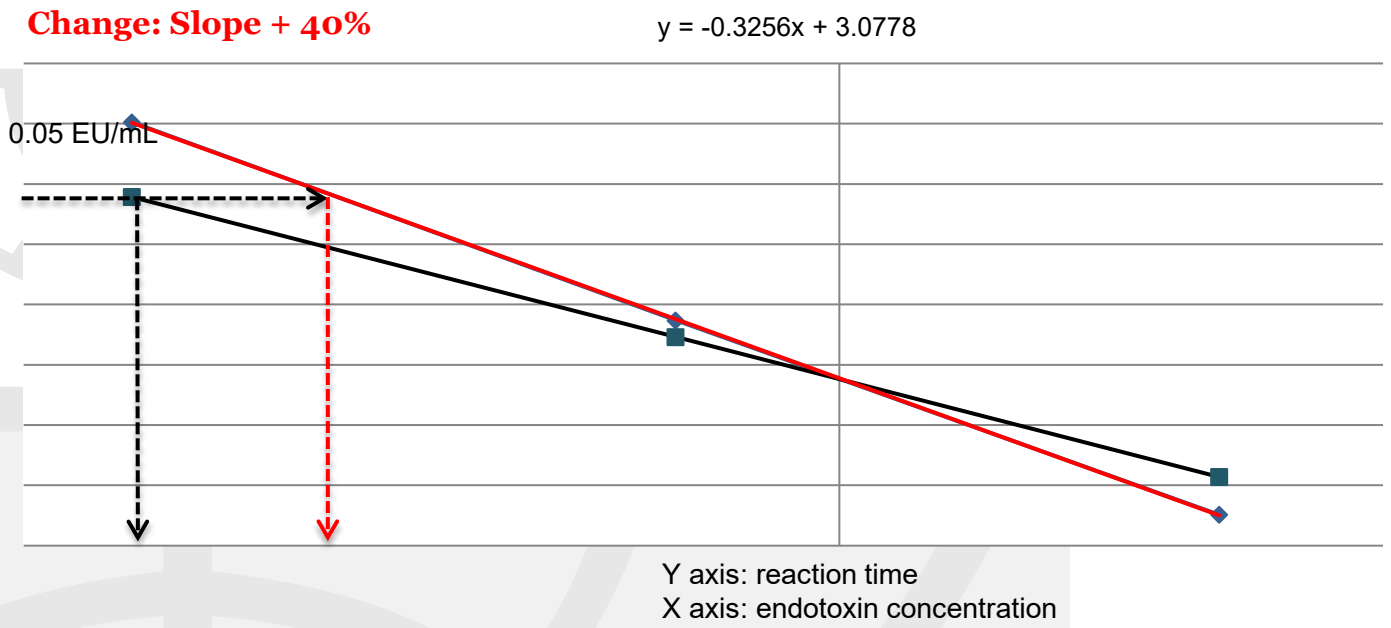
Benchmark curve:

EU/mL	Onset (seconds)
5.0	882
0.5	1500
0.05	2575

882 seconds reaction time for an unknown = 5.0 EU/mL on the benchmark curve (black)
820 seconds reaction time for an unknown = 6.8 EU/mL on the modified curve (red)

Difference = 136% of nominal value

Dissecting the Standard Curve: Slope



Benchmark curve:

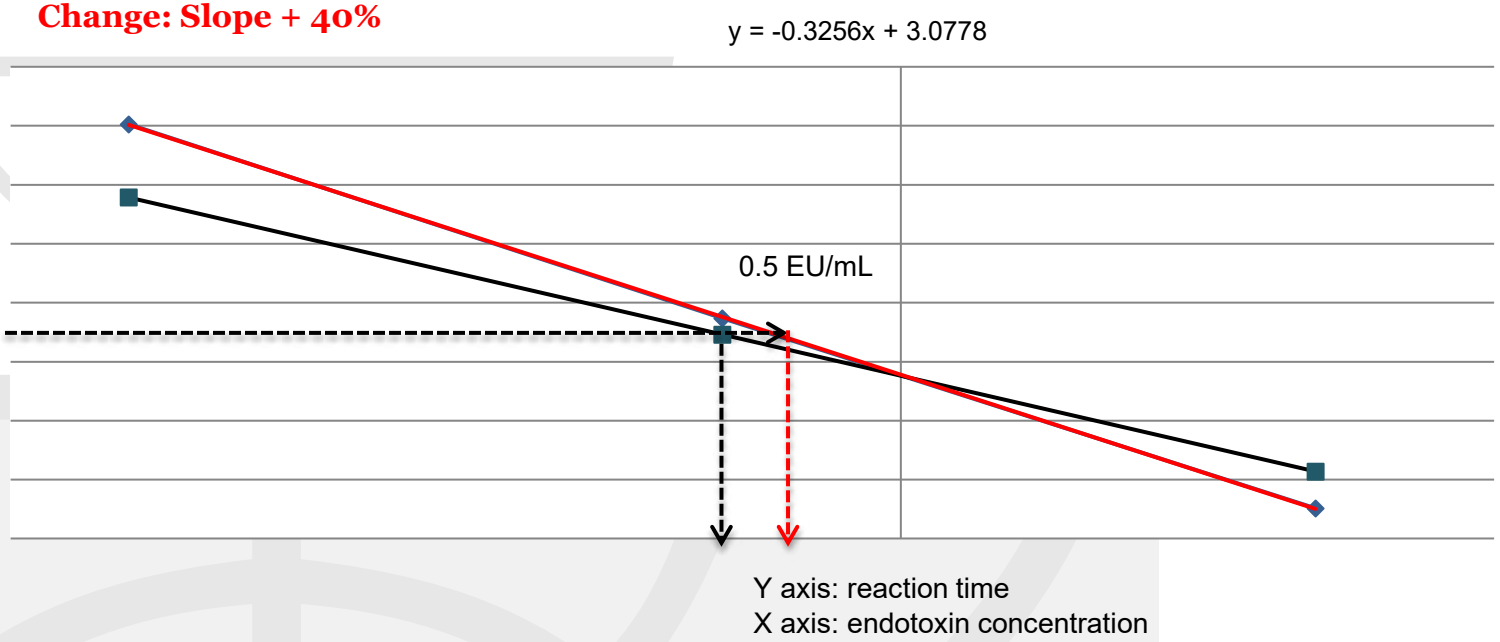
EU/mL	Onset (seconds)
5.0	882
0.5	1500
0.05	2575

2390 seconds reaction time for an unknown = 0.05 EU/mL on the benchmark curve (black)
2390 seconds reaction time for an unknown = 0.119 EU/mL on the modified curve (red)

Difference = 239% of nominal value.
If the action limit for your WFI is 0.05 EU/mL, what is the implication of this modified curve?

Dissecting the Standard Curve: Slope

Change: Slope + 40%



Benchmark curve:

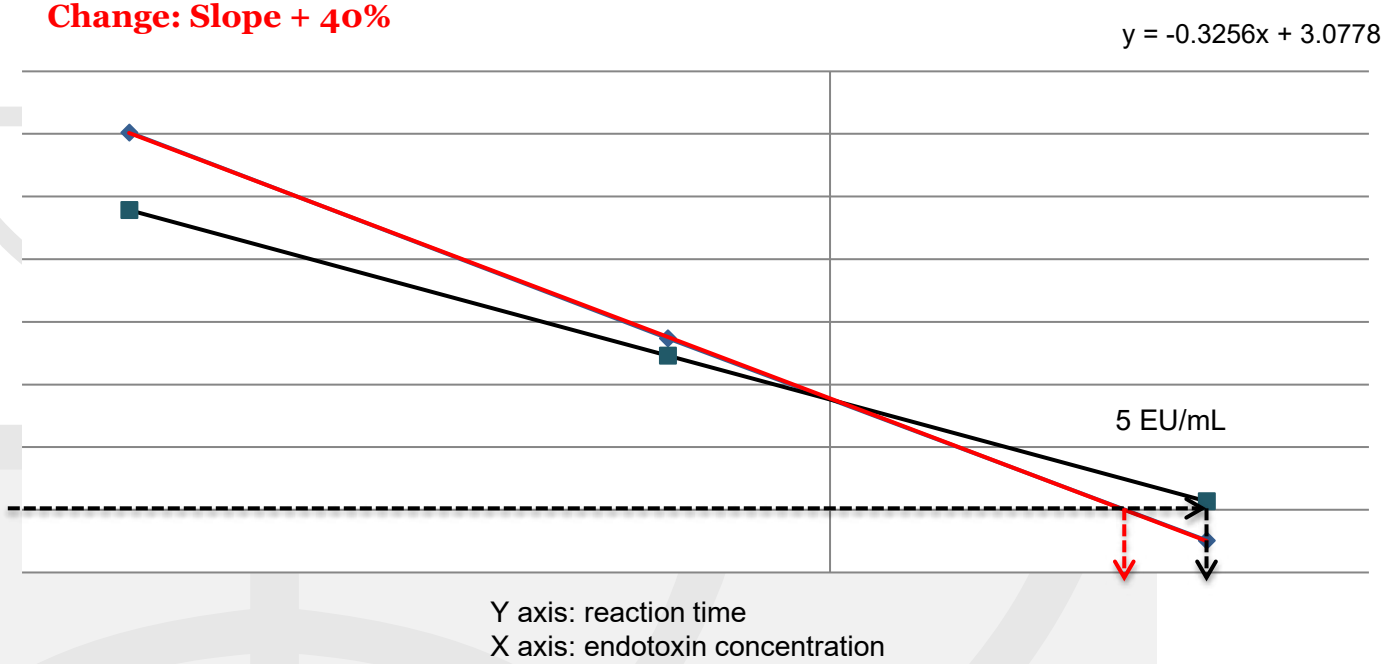
EU/mL	Onset (seconds)
5.0	882
0.5	1500
0.05	2575

1492 seconds reaction time for an unknown = 0.5 EU/mL on the benchmark curve (green)
1400 seconds reaction time for an unknown = 0.62 EU/mL on the modified curve (orange)

Difference = 124% of nominal value

Dissecting the Standard Curve: Slope

Change: Slope + 40%



Benchmark curve:

EU/mL	Onset (seconds)
5.0	882
0.5	1500
0.05	2575

710 seconds reaction time for an unknown = 5.0 EU/mL on the benchmark curve (black)
820 seconds reaction time for an unknown = 3.2 EU/mL on the modified curve (red)

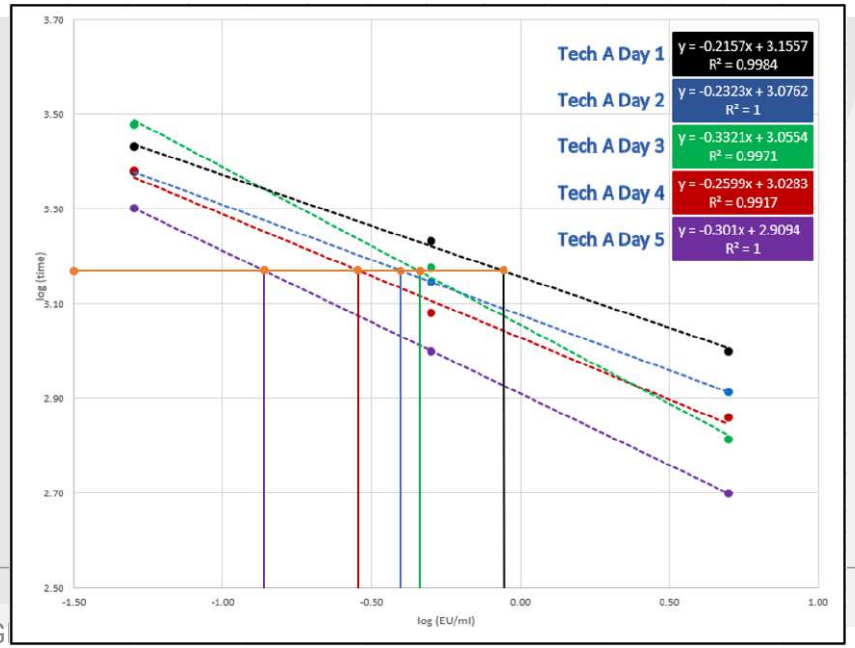
Difference = -64% of nominal value

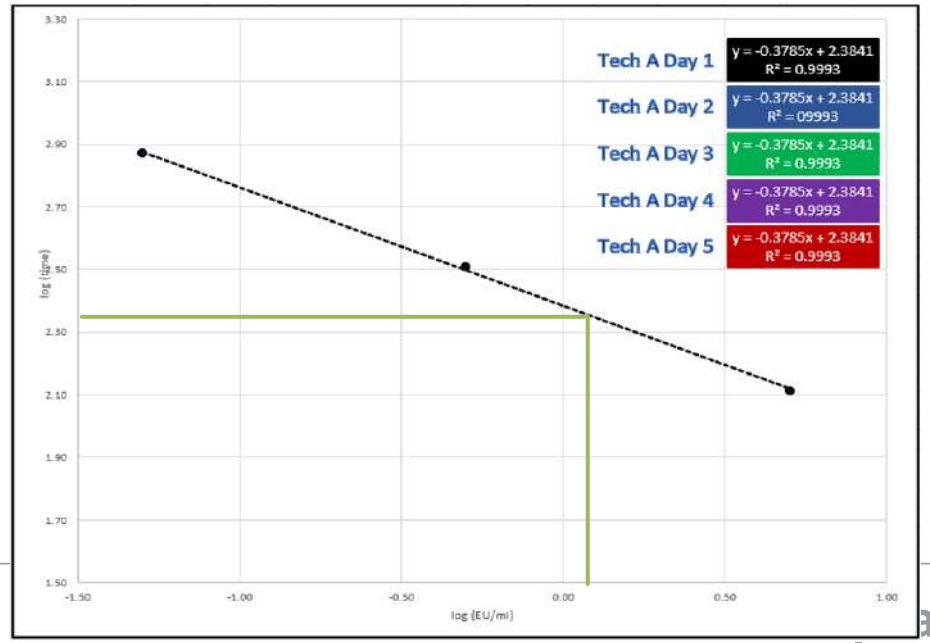
Dissecting the Standard Curve: Slope

Is R value sufficient to access the quality of Standard Curve?

Consider establishing a benchmark for monitoring:
Slope, Y intercept, Onset times

Consider Technologies using Archived Standard Curve



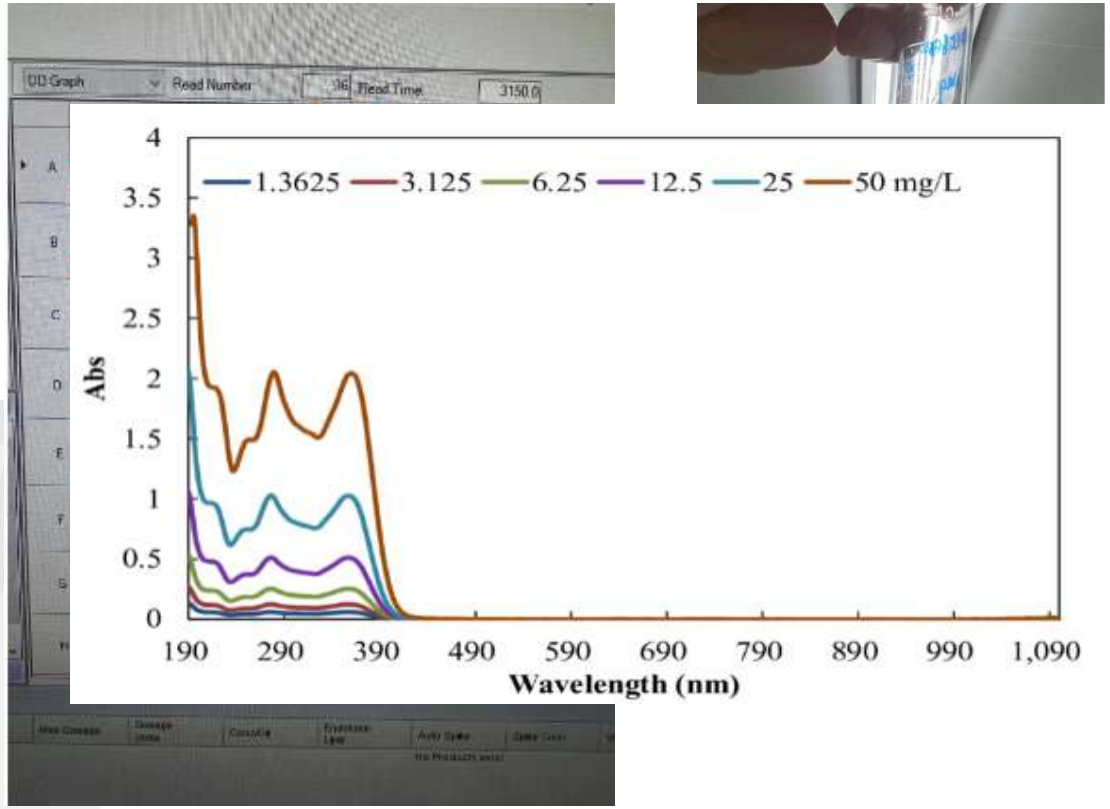


Case Study 4: Sample effects

- Sample Type: Tetracycline Hydrochloride
- Clear but slight yellow-ish
  - Test on Kinetic Turbidimetric assay
- pH of sample adjusted to ~7
- Invalid test (CV% failure)
- Can I look at the plate results?

Case Study 4: Sample effects

- Sample type:
  - Tetracycline Hydrochloride
- Can I look at the plate results?
  - Kinetic Turbidimetric assay
- Absorbs at 340nm
- Solution
  - Kinetic Chromogenic method
  - Dilute further (not exceed Max valid dilution)



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Thank you!
Questions?

LUNCH BREAK

Please return to your seats at 14:00

Putting it all in practice: Group Breakouts

8/17/2023

KNOWLEDGE SHARING

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