Disinfection, Decontamination, Sterilization: **An Introduction**

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- Context (definitions – legislation)
- Chemical disinfection (biocide types – validation)
- Sterilisation (validations)
- Effluent decontamination (Kill tanks, thermal stations)
- Gaseous disinfection

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1. Context

Disinfection, Decontamination, Sterilization =

'basic strategies for treating ***surfaces***, ***items,*** and ***areas in laboratories*** to ***eliminate the possibility of transmission of infectious agents*** to laboratory workers, the general public, and the environment'

Disinfection, Decontamination, Sterilization: what does it mean?

Many definitions!

Disinfection: a process that reduces the number of organisms to a level that is not harmful to health

Sterilization: a process used to render an object free from all viable microorganisms

Decontamination: a process of cleaning, combined with disinfection or sterilization (every day routine)

Legal framework - biocides use

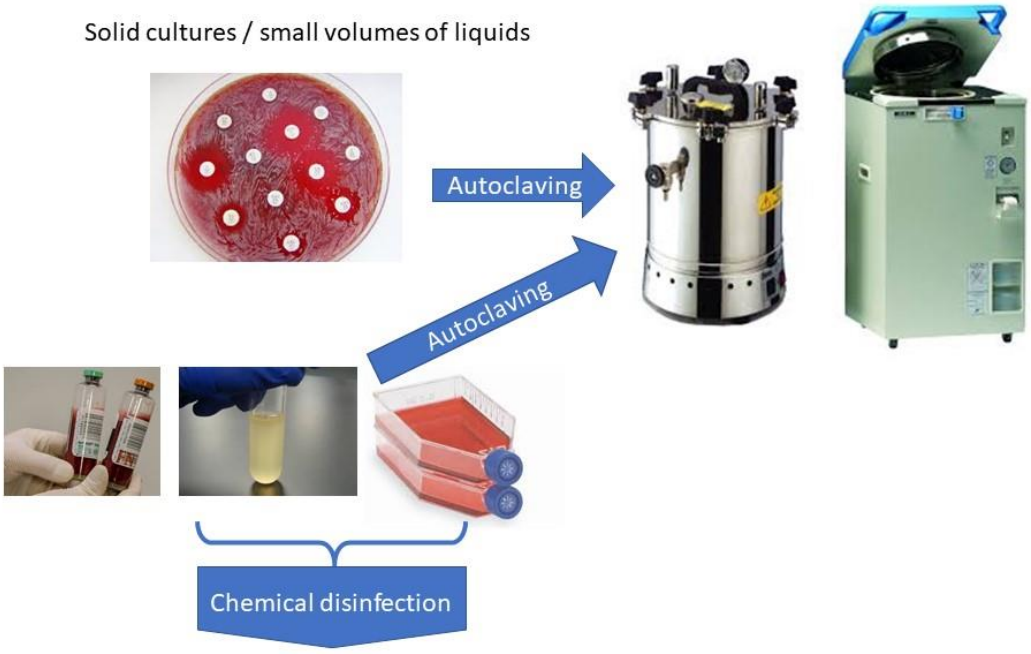
Examples in Europe:

- EU Directives 2000/54/EC on the **protection of workers** from risks related to exposure to biological agents at work
- Biocidal Products Regulation, Regulation (EU) 528/2012

At the international level:

Laboratory Biosafety Manual, 4th edition. World Health Organization (WHO) (2020).

To each situation, its way of decontamination



To each situation, its way of decontamination

Effluents decontamination

Decontamination SINK

Treatment in kill tank

To each situation, its way of decontamination

Biosafety Cabinets (Microbiological Safety Cabinets)

Routine decontamination of work surfaces (chemical disinfection)

Gaseous disinfections of work area, the plenum, the HEPA filter (**integral disinfection** before servicing)

To each situation, its way of decontamination

Integral Gaseous disinfection of the laboratory (BSL3-4)

2. Chemical disinfection

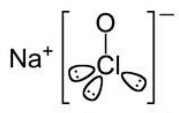
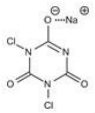
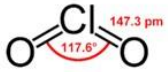
1) Disinfectants types

2) Assessment of biocide properties using biological test method (validation)

1) Disinfectants types ('Biocides')

- Chlorine releasing agents
- Alcohols
- Phenolics
- Aldehydes
- Quaternary Ammonium compounds (QAC)
- Iodates

Chlorine releasing agents:
Hypochlorites and Chlorine dioxide

 NaClO (Bleach)  NaDCC (solid)	
Parts per million (ppm) of available chlorine (av. Cl)	
Surface disinfections: 1 000 ppm av. Cl[1]	
Discard jars: 2 500 ppm av. Cl[2]	
Blood/Body fluids: 10 000 ppm av. Cl[3]	
Organisms producing spores (ex. Anthrax): 10 000 ppm av. Cl	
Inexpensive AND wide spectrum Suitable for disinfection of liquid wastes Suitable for spill clearance	wide spectrum Less sensitive to organic matters than hypochlorite
Chem instability, Inactivated by matrix corrosive Chlorine fumes	• more expensive • lower title in av. Cl (200-500 ppm) than hypochlorite

1-2-3 = Correspondance to stock (10-11%): 1=1%, 2=2,5% and 3=10%

Alcohols in 70% aqueous solution

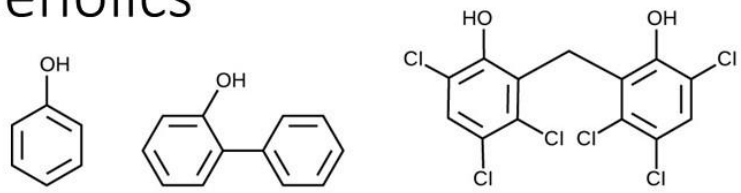
Common use in laboratories:

- Industrial Methylated Spirits (synonyms: Denatured Ethanol, Ethyl alcohol, IMS)
- Isopropyl alcohol (synonyms: Isopropanol, 2-Propanol, Propan-2-ol, IPA)

- Optimum effectiveness at 60-70%
- Rapid **disinfectant of clean surfaces** where corrosion must be avoided
- Used to disinfect hands (hand-rub format)

- INEFFECTIVE against Bacterial spores, mycobacteria, non-enveloped viruses
- RISK of COMBUSTION / FIRE in contact with heat source: use strictly limited to the use of wipe or hand-rubs
- TOXIC HAZARD to staff

Phenolics



Phenol Phenolic compounds (o-Phenylphenol and Hexachlorophene)

Thymol and eucalyptol occur naturally in plants. Other phenolics can be derived from creosote, a component of coal tar.

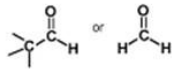
Example of phenolic compound: Triclosan is now commonly used in hand soaps

- Wide range of bactericidal activity (protein denaturation / membrane disruption)
- Not really inactivated by organic matter (suitable for liquid waste disinfection)

- INEFFECTIVE against Bacterial spores
- TOXIC HAZARD to staff (can cause neurological problems; avoid skin and eye contact), especially for phenol sensitive individuals

Aldehydes

Formaldehyde – Formalin - Glutaraldehyde

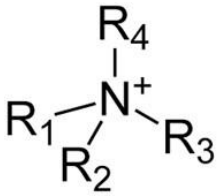


- Mainly used for gaseous disinfection of Biosafety Cabinets
- Formaldehyde can be used for gaseous disinfection of laboratory (with many safety measure to avoid exposure of staff).

- TOXIC HAZARD to staff (highly volatile; toxic and irritant). FORBIDEN IN FRANCE (**will probably be removed in many countries in the near future**)

Quaternary Ammonium (Amines) Compounds 'QACs'

Examples:
Benzalkonium chloride; cetylpyridinium chloride



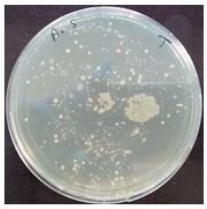
- ***'Certain QACs'*** present a wide range of bactericidal activity (protein denaturation / membrane disruption)
- ***'Certain QACs'*** are effective against non-enveloped viruses

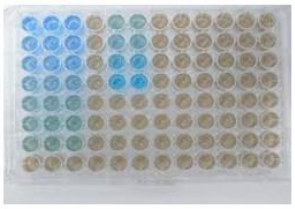
- Can appear good in test but often unreliable in practice (effect of pH)
- Readily inactivated by organic matter (surface disinfection)

2) Assessment of biocide properties using biological test method

Principle of the method:

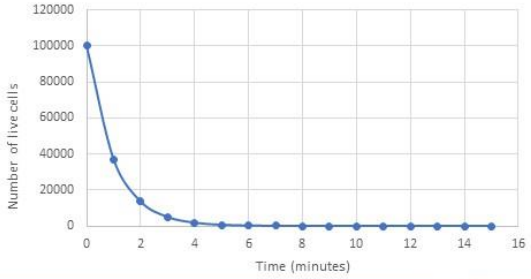
1) measure the cell viability (CFU counting, enzyme assay, …) at different treatment times



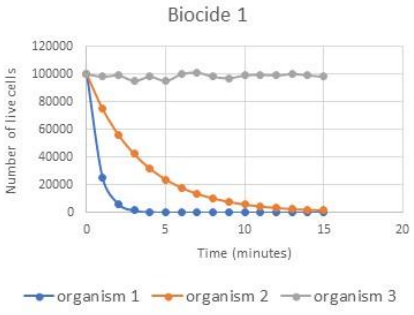


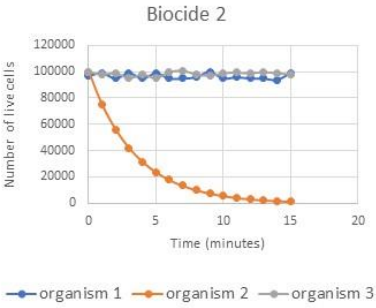
Metabolic activities assessed by Colorimetry (multiwell plate)

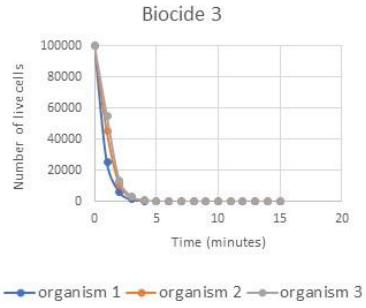
2) Establish the mortality kinetic using the biocide X:



Comparison of 3 biocides, to neutralize 3 µ-organisms

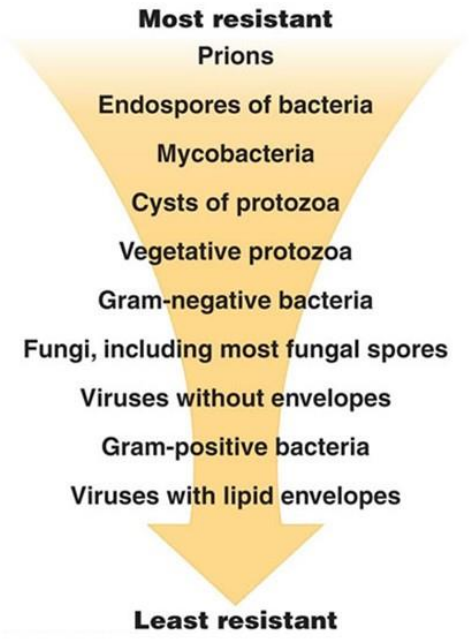






Only Biocide 3 is effective all three organisms

Organisms do not present the same sensitivity towards biocides



Another way to state it:

Biocides have not the same disinfection power

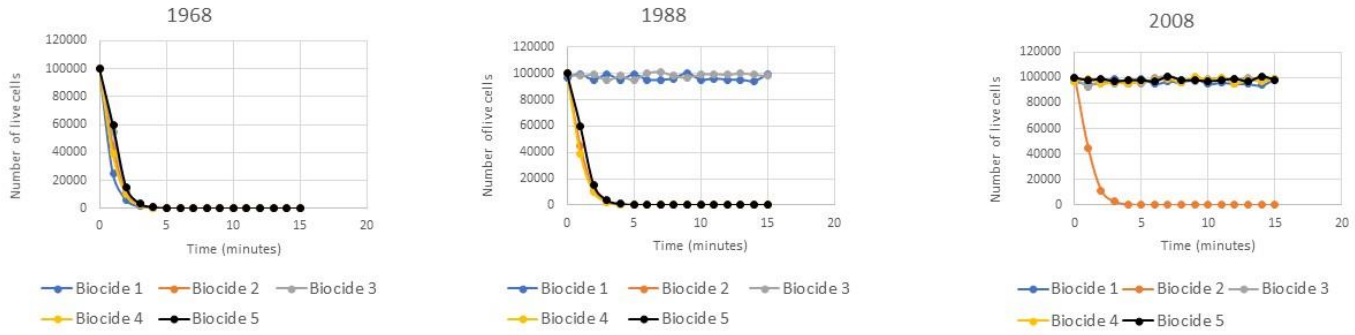
Disinfectant	Efficacy on				
	Fungi	Bacteria	Mycobacteria	Spores	Virus
Ethanol 70%	-	++	++	-	+/-
Hypochlorite (10%)	+	++	+	+	+
Formaldehyde (carcinogenic!)	++	++	++	++	+
Hydrogène Peroxide 6%	+	++	+	+	+
Quaternary ammonium compounds (QAC) *	++	++	++	++	++

PRION – only 1N NaOH is effective!

The choice of the suitable biocide is based on tis validation, especially for CAQ

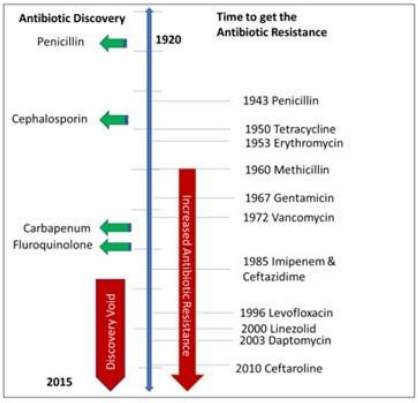
Quiz – question # 1

Comparison of biocide efficacy over time



In 2008, only Biocide 2 is still effective

Organisms could develop resistance to biocides:



It can be anticipated that antibiotic resistance will continue to develop more rapidly than new agents to treat these infections become available

The World Health Organization (WHO) has classified antibiotic resistance as one of the greatest global threats to human health by warning that a "post-antibiotic era", where common infections and minor injuries can kill, may soon be a reality

- *Staphylococcus aureus* (MRSA)
- Multidrug-resistant (MDR) *Mycobacterium tuberculosis*,
- beta-lactamase (ESBLs)-producing bacteria

have become a major global healthcare problem in the 21st century

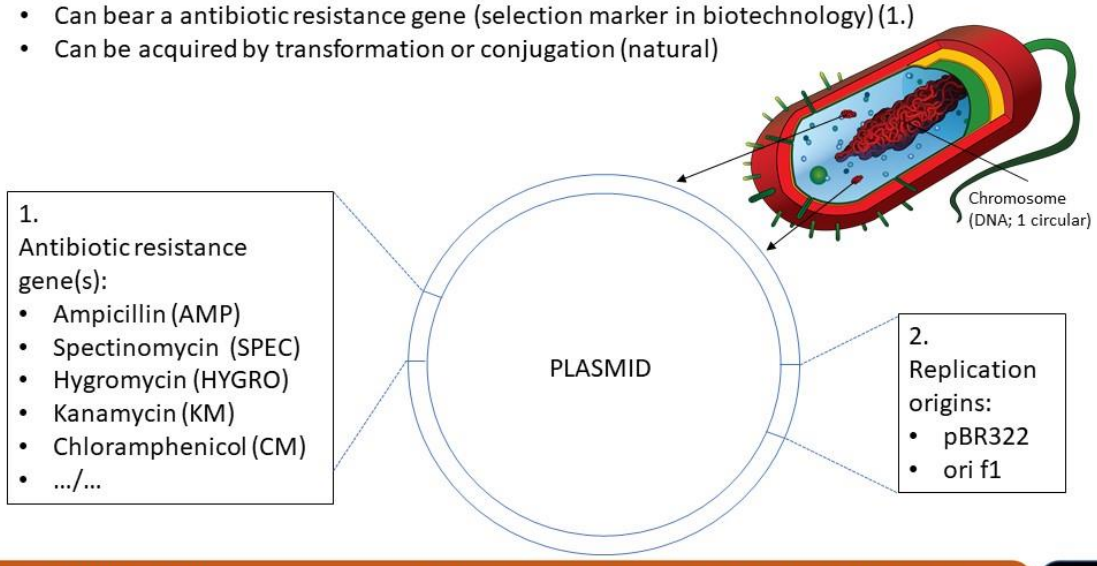
Medina & Pieper, Curr Top Microbiol Immunol. 2016;398:3-33. doi: 10.1007/82_2016_492.

Possible impact of incomplete destruction of µ-organisms by biocides on the transfer of antibiotic-resistance genes?

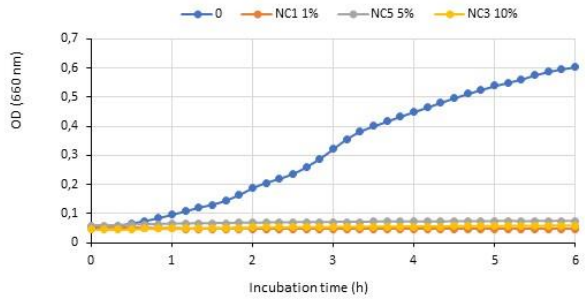
What is the DNase effect of biocide on plasmids?

PLASMID: circular DNA

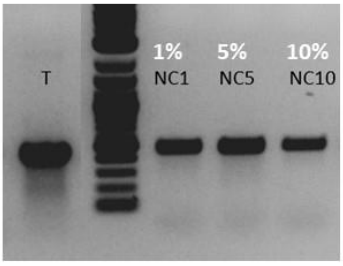
- Autoreplicative (2.)
- Can bear a antibiotic resistance gene (selection marker in biotechnology) (1.)
- Can be acquired by transformation or conjugation (natural)



Certain biocides have no or limited DNase effect



Biocidal effect



No DNase effect

Effect of NaClO treatment (NC, added to culture as 1-10% v:v) of laboratory *E. coli* on:

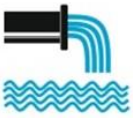
(A) cell growth assessed by optical density (OD) increase with time: the growth is inhibited in all treated cultures (STRONG BIOCIDE EFFECT), compared to negative control (0)

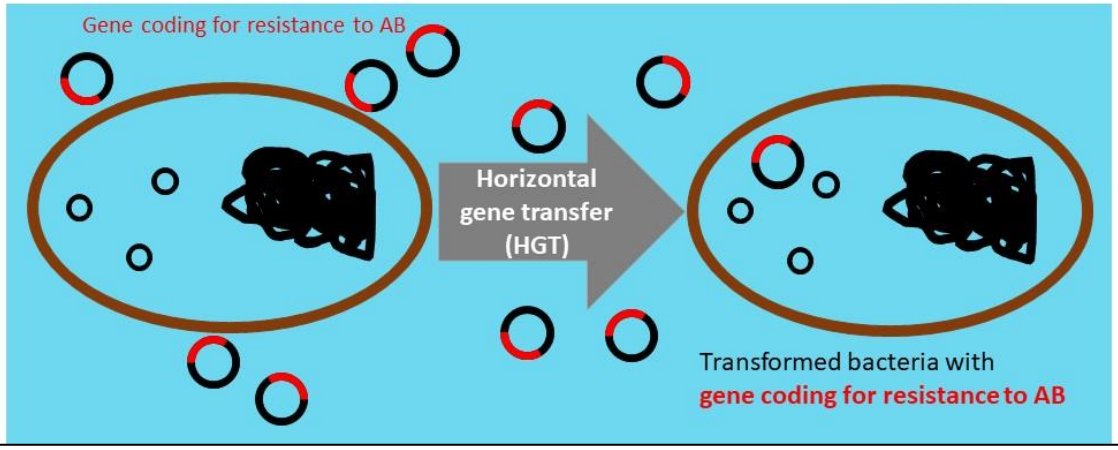
(B) ABR gene integrity assessed using PCR: no (1-5% NaClO) or only LIMITED DNase EFFECT (10% NaClO) in treated cultures, compared to negative control (T)

In spite of the effective killing of organism (effective disinfection), **DNA – including plasmids - could be preserved**

What is the problem?

If treated cultures are released in sewage,
intact plasmids can be taken by naturally competent bacteria (**transformation**)



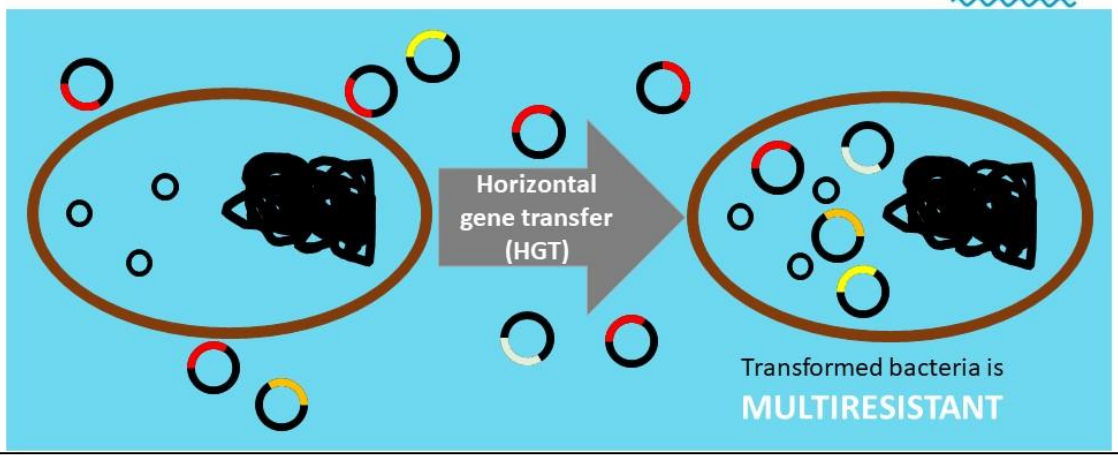


In spite of the effective killing of organism (effective disinfection),

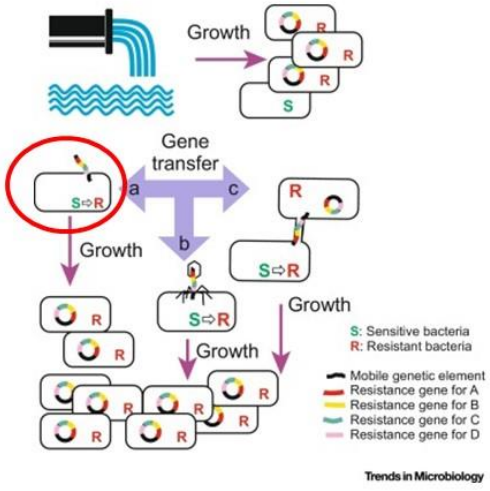
DNA – including plasmids - could be preserved

What is the other problem?

Transformation could occur several times (with ≠ plasmids)
Example with 4 resistance genes



Why care about the accidental release of plasmids in WW?



Accidentally released PLASMIDS from research laboratories could transmit resistant constructs to sensitive bacteria by transformation of « competent » sensitive bactéria

Karkman et al., Trends Microbiol. 2018 Mar;26(3):220-228. doi: 10.1016/j.tim.2017.09.005.

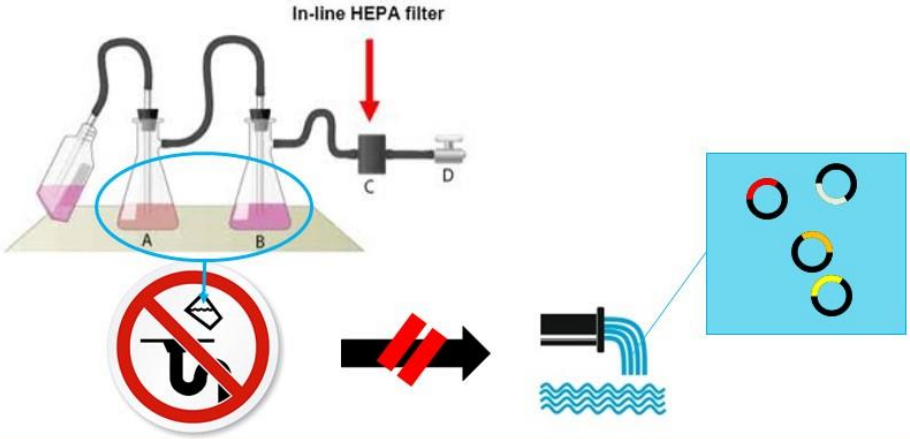
CONCLUSION:

Even after chemical disinfection

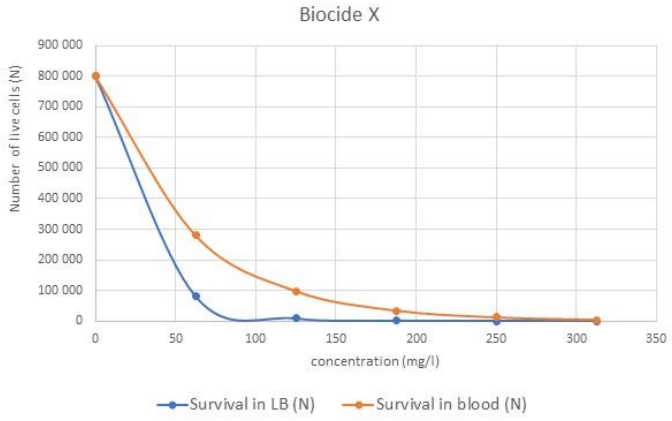
DO NOT POUR treated cultures of pathogenic organisms

(or GM with plasmid coding for AB resistance) **in the sink**

Example: culture medium aspirated in bottle pre-filled with NaClO (disinfection)

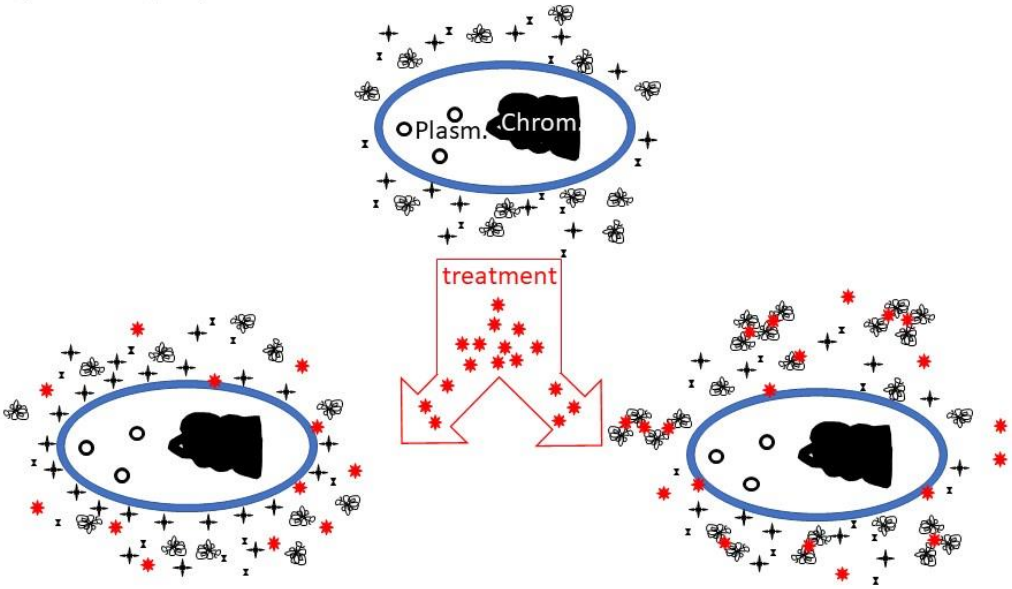


Comparison of biocide efficacy in different media



Biocide X is less effective in blood (matrix effect)

The composition of the medium (matrix) can alter biocide (*) efficacy. Example, bacteria in serum:



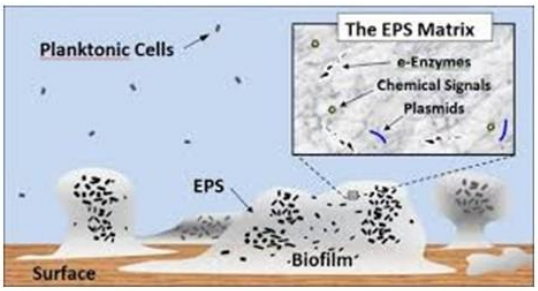
Bacteria protection by matrix

Biocide interaction with matrix

In both cases: reduced access of biocide to its target in Bacteria

The matrix can be created by the bacteria when they aggregate in biofilm.

- Biofilms are formed on the surface of pipes, pipes of VAC systems, respirators, ...
- Biofilms are very difficult to disinfect because of the matrix
- Biofilms are the site of horizontal transfer of plasmids



- Planktonic cells = free bacteria
- EPS: Extracellular polymeric substances:
  - ✓ Lipids
  - ✓ DNA (**including plasmids**)
  - ✓ Polysaccharides
  - ✓ Proteins
  - ✓ Cell debris

2. Steam Sterilisation

A sample of many autoclave models!





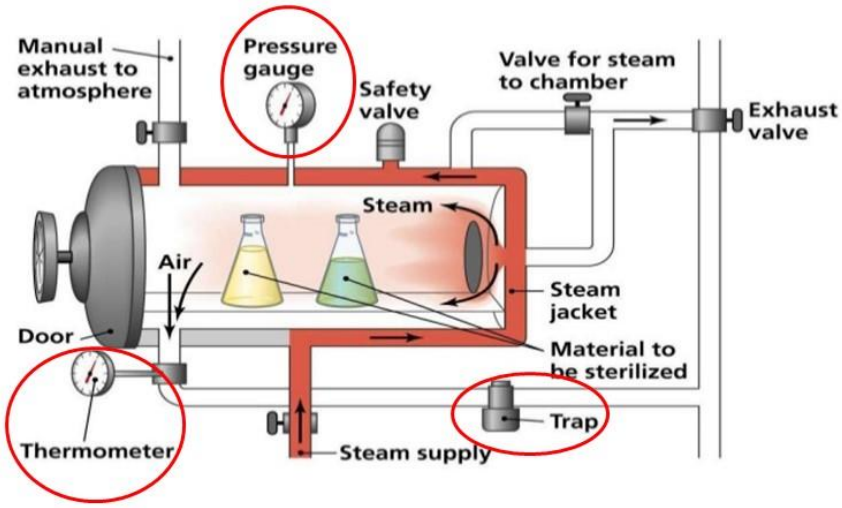
What we try to achieve with steam sterilization:

- Sterilisation of liquid wastes

- Sterilization of solid wastes

Steam sterilization is also applied to prepare sterile material (culture media, pipette tips, …)

Steam sterilization principle



- The temperature and the pressure of the steam should be optimized
- Air should be completely removed
- Recommendation for RG2-RG4 organisms: HEPA filter in the 'Trap'

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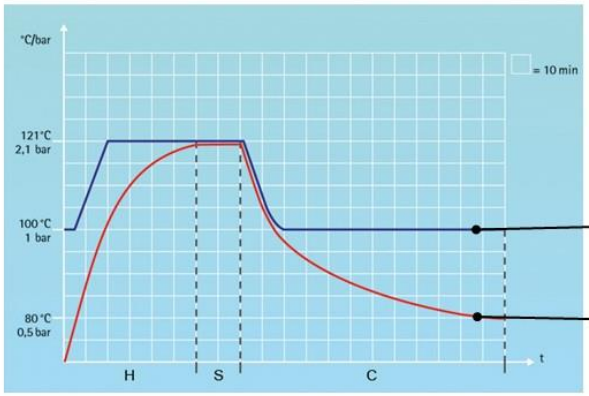
1) Steam sterilization principle

- **Steam sterilization** is carried out at
  - a steam pressure of 2 to 5 bar
  - a sterilization temperature between 100 to 150°C
  - a sterilization time between 3 and 20 minutes
- Commonly used **steam sterilization** parameters are
  - 121°C, 15 to 20 minutes
  - 134°C, 3 to 5 minutes

Relation between Temperature and pressure

Steam temperature (°C)	Absolute Pressure (kPA)	Absolute Pressure (bar)
121	200	2
134	300	3
145	400	4
150	500	5

Sterilization process: temperature and pressure profiles



H: Heat-up
S: Sterilization
C: Cooling

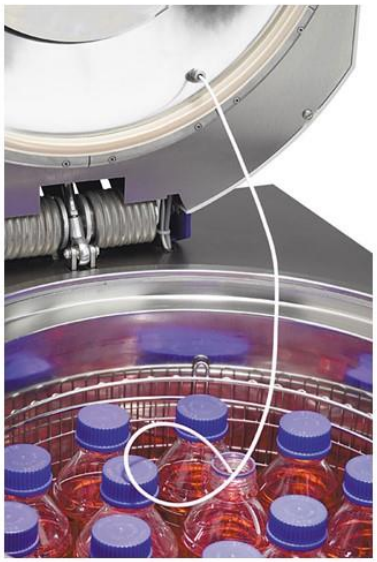
Pressure (bar)

Temperature (°C)

Use temperature sensors to monitor the heating of items

Testing:

each box and bottles contain a temperature sensor



Routine sterilization:

one item is sacrificed for the monitoring of temperature.

Quiz – question # 2

Quiz – question # 3

2) Validation of Autoclave (DQ-IQ-OQ-PQ)

Different types of Validation:

DQ - Design Qualification
IQ - Installation Qualification
OQ - Operational Qualification (without products)
PQ - Performance qualification (with products)

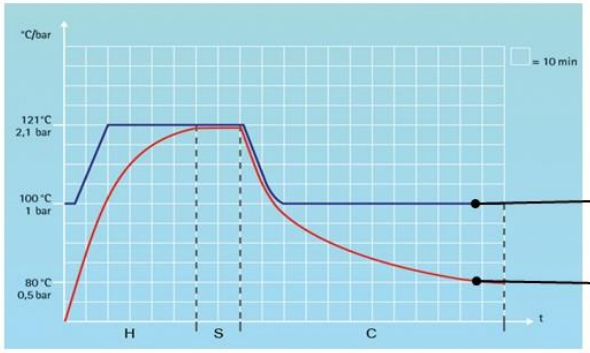
Manufacturer / Distributor

USER

All results of the tests carried out must be documented (test plan)

Operational qualification (OQ)

- Check autoclave components
- Check the process (Temperature and Pressure profiles) :



H: Heat-up
S: Sterilization
C: Cooling

Pressure (bar)

Temperature (°C)

DURING 15 MINUTES

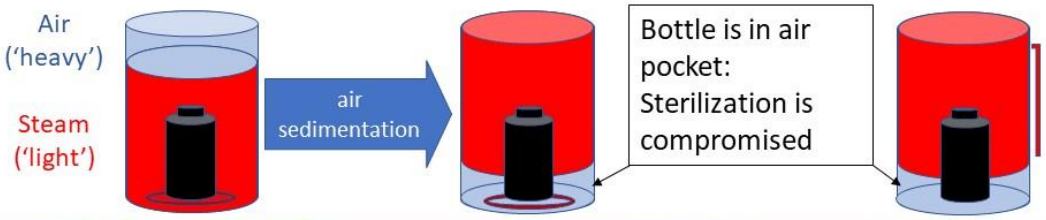
Temperature indicator tape

Can not be used as a marker of validation
(no precision of the duration)

Two ways to generate steam

- A: by internal heating elements
- B: by separate steam generator

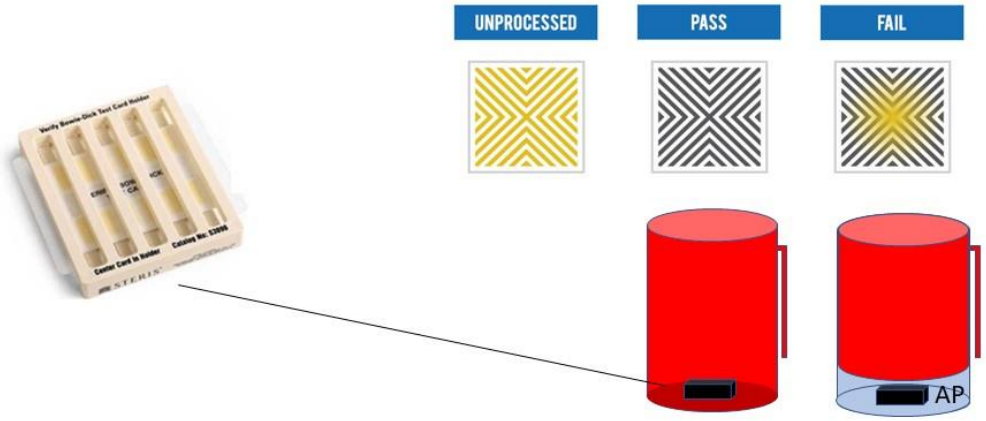
AVOID AIR POCKET IN THE STERILIZATION CHAMBER
Examples of incomplete filling of the chamber:



Operational qualification (OQ) - EN 13060

How to detect 'Air pockets' (AP):

BOWIE-DICK Test (no samples in the chamber)



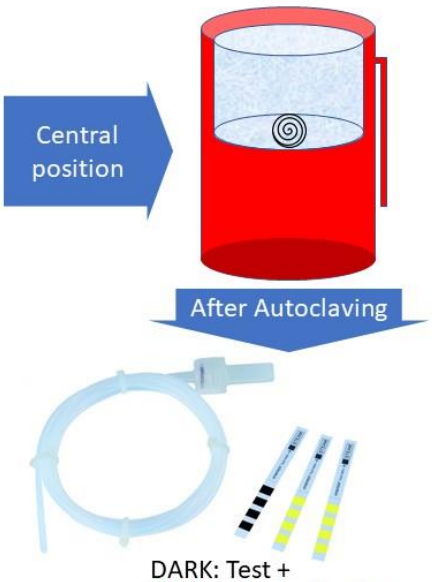
Operational qualification (OQ)

Steam penetration test (when processing wrapped or hollow loads)

HELIX Test (no samples in the chamber)

Steam sensitive indicator introduced in a tube

Tube connected tightly to a capillary



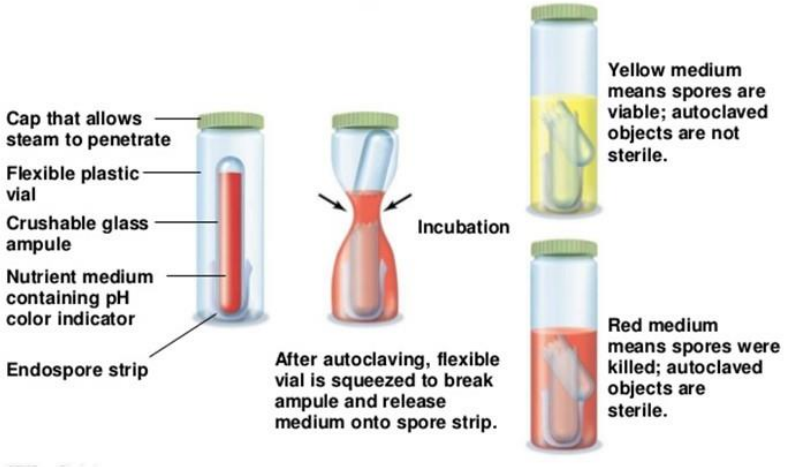
DARK: Test +

Yellow: Test – (maintenance needed)

Quiz – question # 4

A functional test of sterilization is needed!

Use of biological indicators of sterilization.
Example of a commercial test



The biological test should be validated

2) Validation of Sterilization (Biological indicator)

Determination of STERILITY ASSURANCE LEVEL (SAL)

SAL is used to express the probability of the survival at the end of the sterilization process.

SAL of $1/10^6$ indicates a 1 in 1,000,000 likelihood of an organism surviving to the end of the sterilization process.

The sterilisation conditions should be adapted to reach this probability

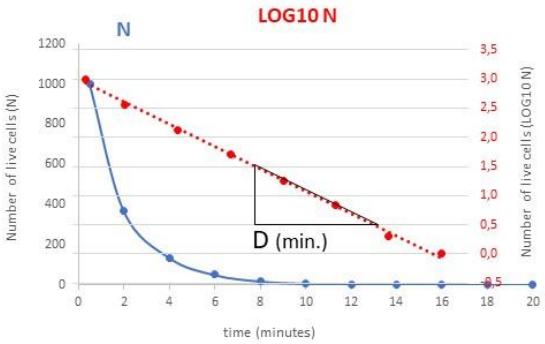
SAL is based on the Dvalue

Time unit needed to obtain a 90% decrease the organism number (example, from 10 000 to 1000)

The mortality kinetic curve (N, cell count over time) is not appropriate for accurate determination of D

linearization

LOG10 N over time
Time needed for a 1 LOG decrease = D



Information given by the Dvalue:

- *it is directly proportional to the body's resistance to sterilization*
- *it makes it possible to determine the time necessary to reach the Sterility Assurance Level ("Log6" principle)*

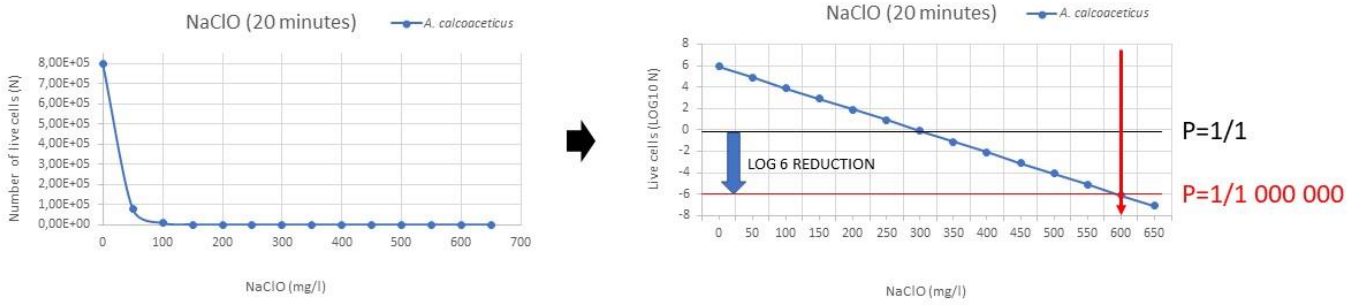
Quiz – question # 5

SAL Determination Method

1. **Determine D value** (in minutes, mg/ml, %, ppm, moles, … depending on the treatment)
2. **Evaluate n** = number of D values needed to kill all cells but 1 (to reach '0' on the LOG10 scale):
   the likelihood (P) to restart a culture is 1 in 1 part or 'P = 1/1'
3. **Add 6*D values** (= 6 log reduction) to diminish the likelihood to 1 cell alive in 1 000 000 parts or 'P = 1/1 000 000'

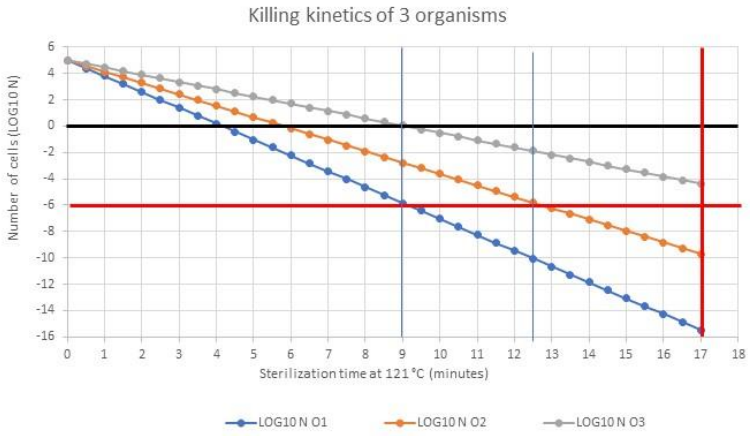
SAL is reached with (n+6)*D value in minutes, mg/ml, %, ppm, moles, … depending on the treatment

Example for *A. calcoaceticus* treated with NaClO 5bleach)
D=50 mg/l , n=6, SAL Dose of NaClO = (6+6)*50 mg/l = 600 mg/l



Examples of SAL values for three µ-organisms

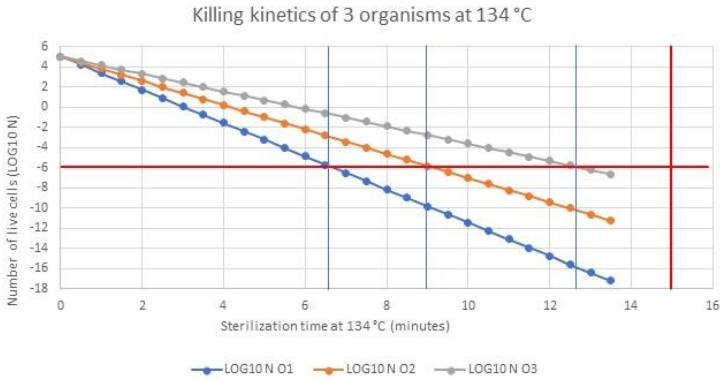
1) The autoclave (process at 121 °C, 17 minutes) kill all organisms (live cell counting)
2) BUT, SAL is not reached for the the 3 organisms after 17 minutes of sterilisation



SAL-O1: 9 minutes
SAL-O2: 12,5 minutes
SAL-O3 > 18 minutes

Another example (100 000 cells/ml in cultures of 3 µ-organisms)

Steam sterilization at 134°C : D is reduced for all organisms

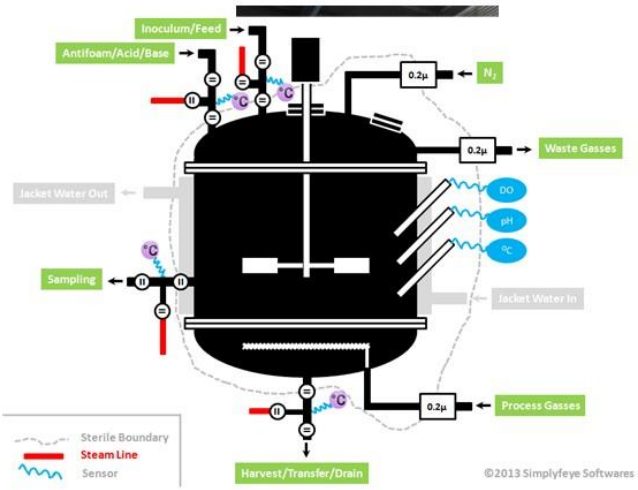


SAL-O1: 6,7 minutes
SAL-O2: 9,1 minutes
SAL-O3: 12,8 minutes

In these conditions, SAL is reached in 13 minutes for all organisms

3. Effluent Decontamination Systems (EDS)

Sink decontamination system
(recommended in BSL3 and BSL4)



Biofermenter:
can also act as a « kill tank » (steam sterilization)

Thermal decontamination in the kill tank: batch system (steam/°C and/or chemical dis.)

Steam/°C

Storage tank (chem. dis.)

The decontamination method must be validated

Use of killing tanks present a few challenges !

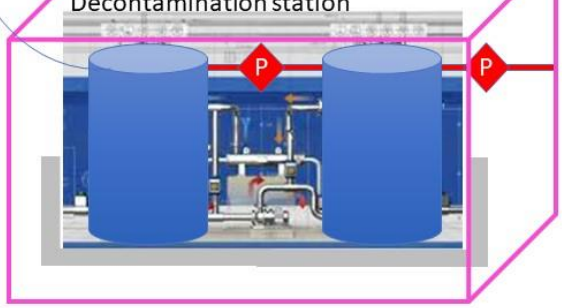
Fermenter decontamination (SOP – Official report)

Waste duct:
Double wall
Leak detection
Inspection ditch

! **FMD** oubreak in UK due to **faulty drainage pipe**

CONTAINED (level 2, 3)

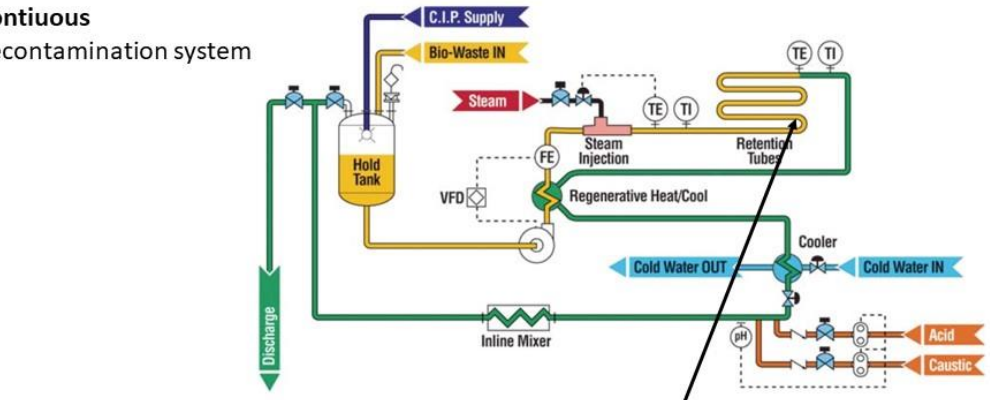
Decontamination station



Impede spontaneous emptying of tanks:
- use a pump (P)
- SOP for the emptying
- Official report

Also, consider environmental issues (chemical neutralization on effluents, …)

Contiuous
decontamination system



In retention tubes: steam and 121°C, 20 minutes

The final liquid waste should be processed in a waste water treatment plant

4. Gaseous sterilization

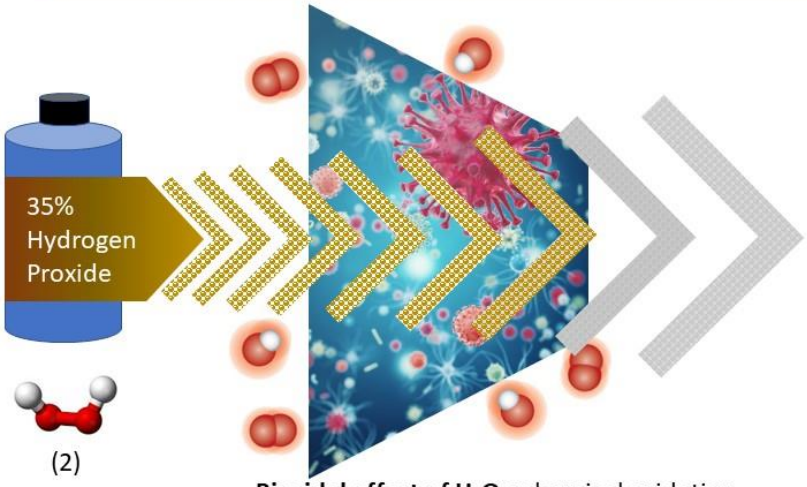
Use of air as a vehicle for diffusing, to all exposed surfaces of the room and its content, a gaseous biocide.

Examples:

Biocide		Pro's / Con's
Ethylene Oxide (EtO)	C_2H_4O	Con: Safety hazards, long disinfection procedure
Ozone	O_3	Pro: food production Con: irritant for respiratory tract and eye mucosa
Chlorine Dioxide	ClO_2	Pro: does not leave any residue Pro: building remediation (and Hospital room in US) Con: corrosive for material
Formaldehyde	CH_2O	Pro: potent biocide **Cons (many!):** • Necessitates high T° and high relative humidity • many safety hazards (toxic, allergenic, carcinogenic) – Not approved by FDA – Forbidden in France • Formaldehyde residue can remain on goods

Widely used (in biopharmaceutical industries, laboratories and hospitals):

Vaporised Hydrogen Peroxide (H_2O_2) Technology

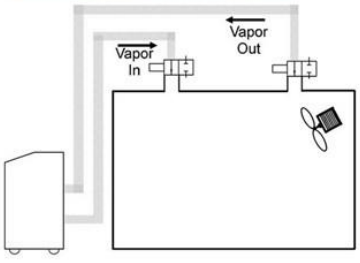


Biocidal effect of H_2O_2: chemical oxidation of cellular components by radicals

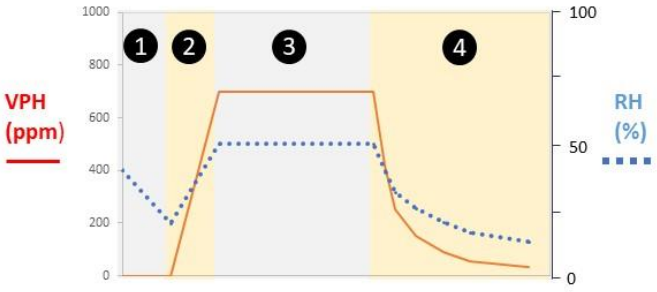
Superoxide $\cdot O_2^-$ Hydroxyl radical $\cdot OH$

Applications of VHP methodology : Room decontamination (BSL3-4)

Example using an external VHP generator



Overview of the decontamination cycle:



1. Dehumidification
2. Conditioning
3. Decontamination
4. Aeration

**control of moisture*

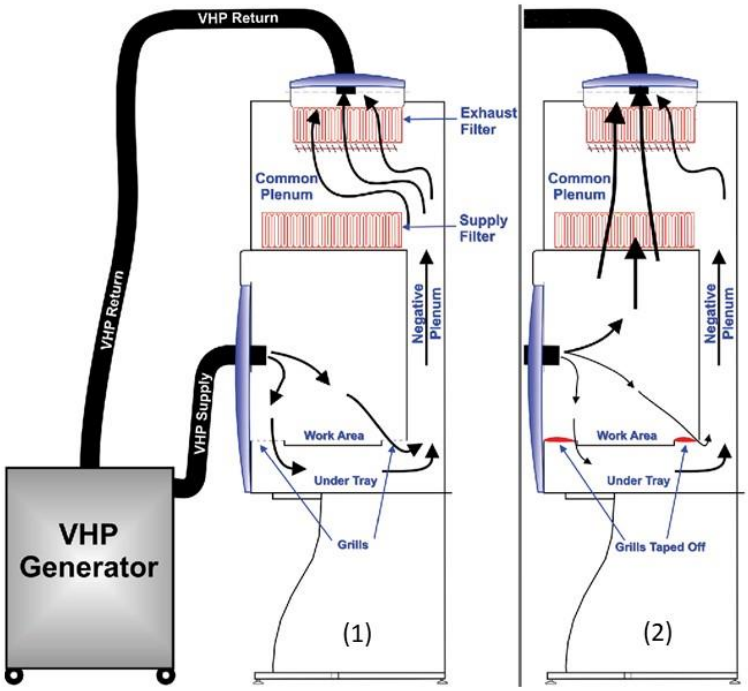
Cycle duration: 5 to 10 hours (depending on the room volume and other factors)

Vaporized H_2O_2 (VHP) is not a gas

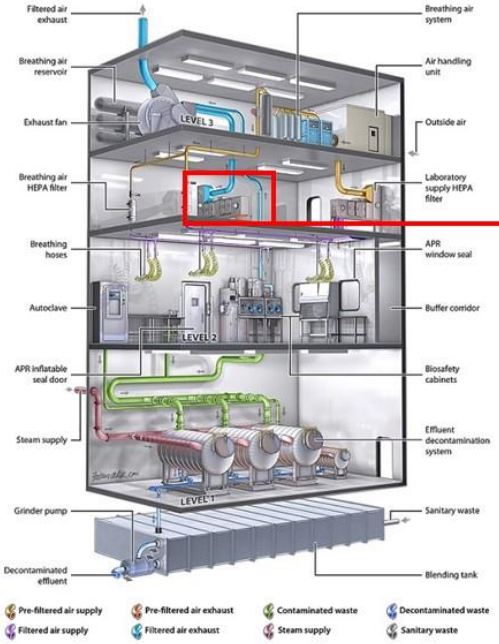
- fan needed for the optimal dispersal of the decontaminating aerosol
- above the saturation point (dew point), **H_2O_2 vapour starts to 'micro-condense'** on surfaces (=decontamination phase)

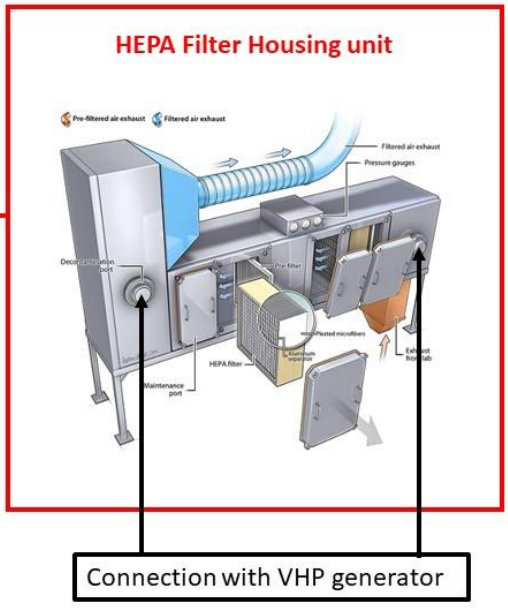
Applications of VHP methodology : BSC HEPA Filters decontamination

Decontamination in two phases:
(1) Exhaust filter
(2) Work protection filter



Applications of VHP methodology : Room HEPA Filters decontamination





Validation of VHP Process

- **Operational qualification (OQ)**
  - Monitoring of HP concentration (ppm)
  - VHP diffusion test

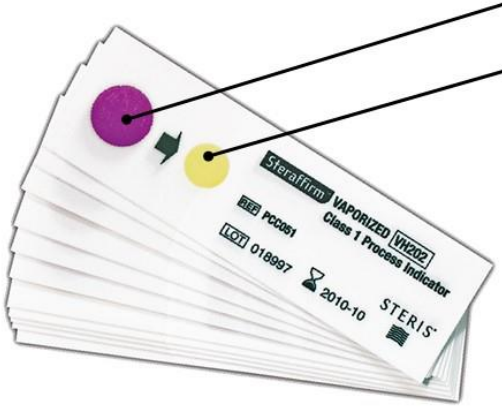
  ALSO: leak test of the room / BSC to be fumigated!

- **Performance qualification (PQ)**

  SAL, using appropriate biological indicators

1) Monitor VHP diffusion

(at different places in the lab)



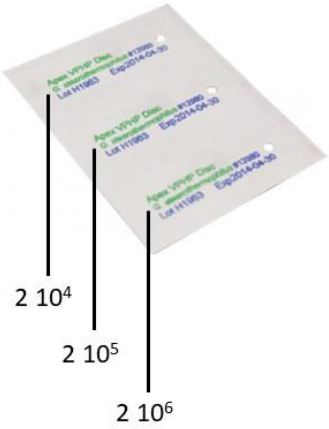
Magenta ink
↓
Yellow at the contact with VHP

1) Monitor cell mortality (biological indicator)

Available commercial BI

Metal disc containing dried
G. stearothermophilus spores
(2.3 X 106/disc)
in VHP permeable Tyvek pouch
(LOG6 reduction)

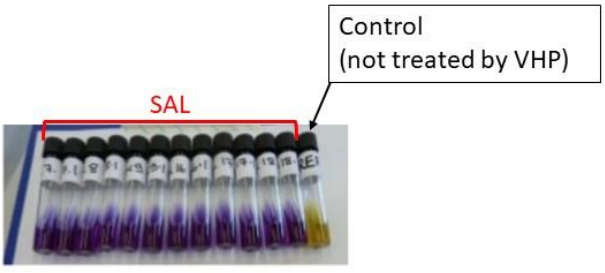
3-concentration system (for assessment of SAL conditions):



At the end of the decontamination cycle:
- Disc are incubated in growth medium
- Cell growth is measured in a biochemical assay

1) Monitor cell mortality (biological indicator)

Available commercial BI



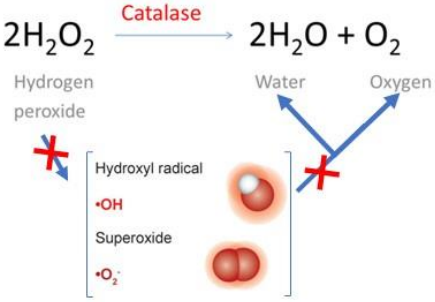
For each BI test,
VHP diffusion test
should be done simultaneously

It is always possible to make ones own BI
BUT its validation is mandatory

H_2O_2 hazards

Hazard	Prevention measures
Causes serious eye damage	**Wait until the concentration has dropped to 1 ppm before accessing the treated room**
Harmful if swallowed	
Harmful if inhaled	
May cause respiratory irritation	
Causes skin irritation	
Carcinogenic (in animals)	
May cause or intensify fire; oxidizer	Keep away from heat/sparks/open flames/hot surfaces

Since H_2O_2 biocide effect is done by $\bullet OH$ and $\bullet O_2^-$,
microorganisms expressing catalase are less or not sensitive



Example:
Meticillin-resistant *S. aureus* (MRSA), produce catalase

Other critical facts about VHP technology

Problems	Needs
H_2O_2 has compatibly issues with some surfaces	The lower the concentration, the less likely damage due to condensation
H_2O_2 can break down in presence of: • galvanized steel • porous substances (paper, wood, untreated block / bricks, …) • Cu and Cu alloys	• Try to avoid this material in the room to be decontaminated • Deposit of biological indicators on stainless steel disc
H_2O_2 is readily absorbed into liquid surfaces	Standing liquid should be avoided

Compatibility issues with some surfaces

Conclusion:

- VHP should be preferred for BSC disinfection
- Another gaseous disinfection should be selected for room decontamination (come back to formaldehyde use?)