



# viable but nonculturable bacteria : Their nature and formation mechanisms with a look in their resuscitation

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Introduction

Characteristics  
of VBNC

Detection

Induction  
conditions

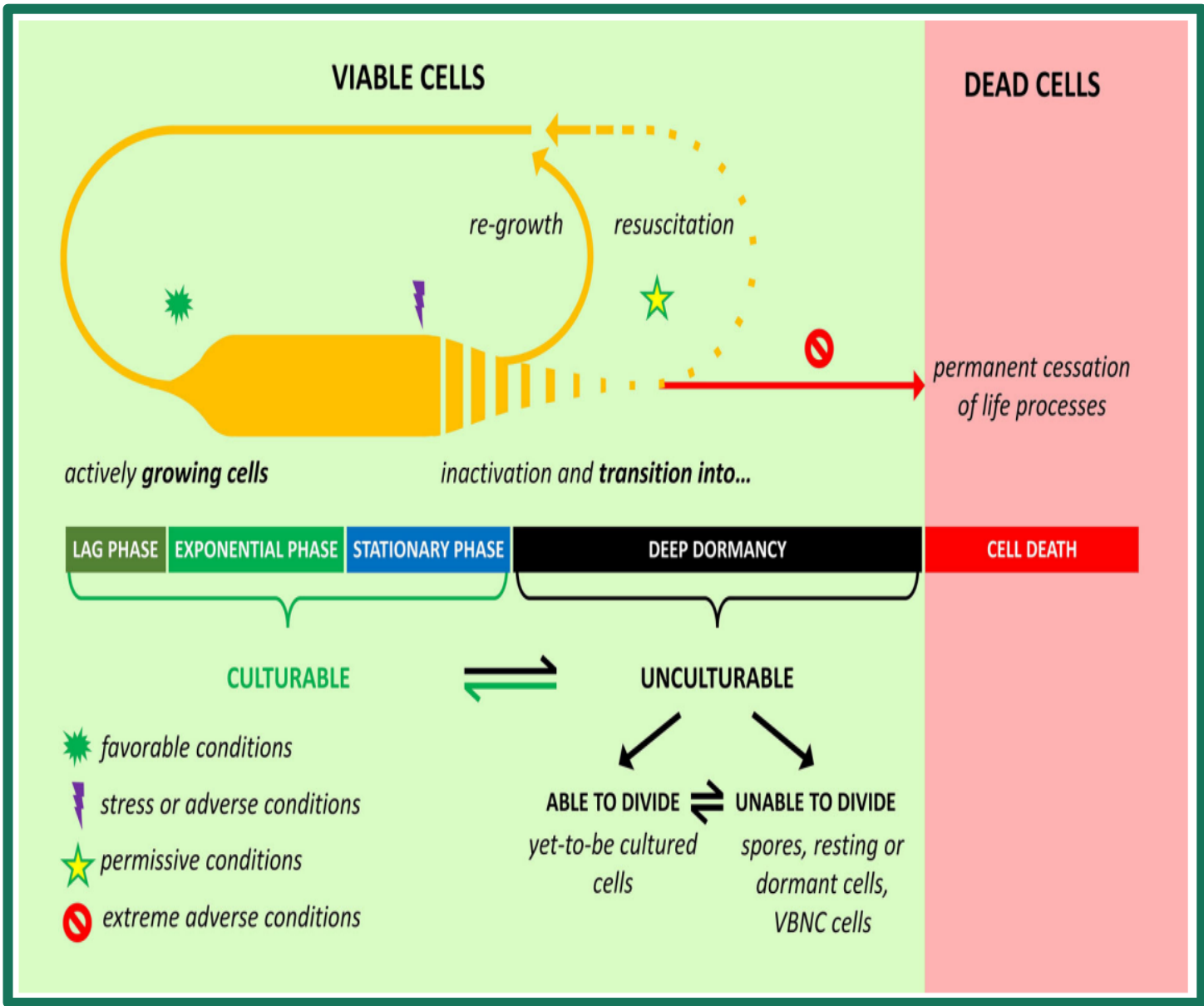
VBNC

Formation  
mechanisms

Bacterial  
resuscitation

Conclusion

Reference



Different physiological states in bacteria ranging from unstressed living cells to dead cells

VBNC cells maintain their viability but unable to grow on routinely-used laboratory media

*E. coli*

*V. cholerae*

*S. enteritidis*

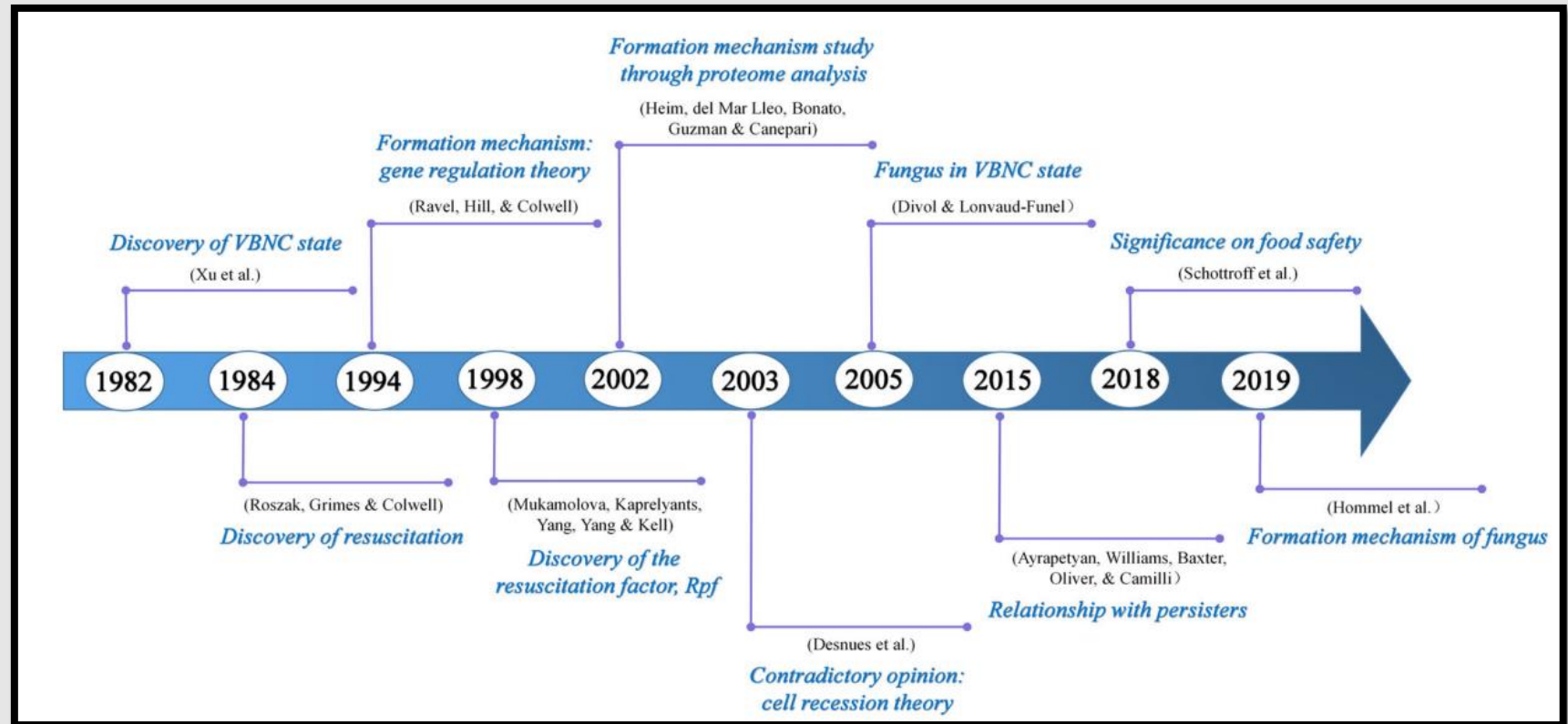
*Cryptococcus neoformans*

omics

To explore the formation or resuscitation mechanisms



Study expression changes of genes or proteins



# Characteristics of viable but nonculturable bacteria

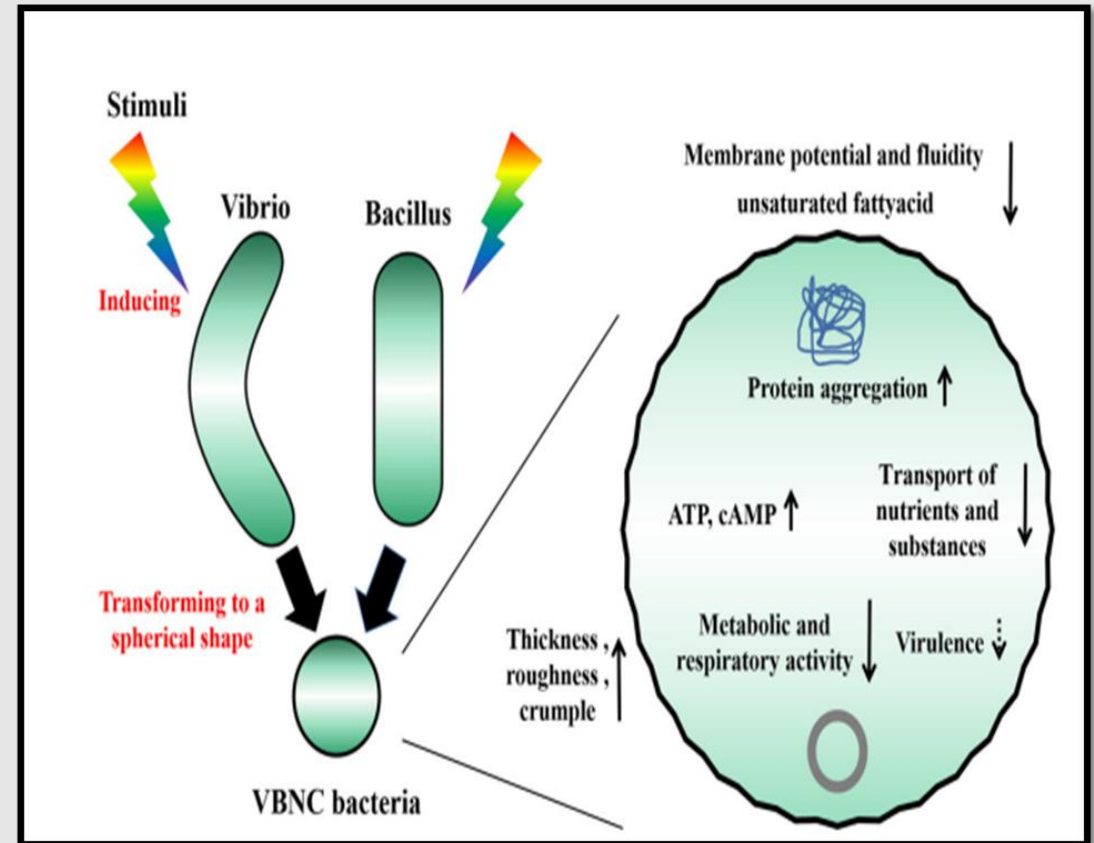


## Variety of bacteria can reportedly induce the VBNC state:

- *Escherichia coli*
- *Campylobacter*
- *Helicobacter pylori*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*
- *Vibrio cholerae*
- *Vibrio vulnificus*
- *Vibrio parahaemolyticus*
- *Salmonella*

# Morphology

- ❑ Bacterial cells typically become small **spherical shape**
- ❑ Significant reduction in bacterial cytoplasm
- ❑ Increase in the thickness of the cell wall



- ❑ Surface becomes rough and unevenly wrinkled
- ❑ Cell wall composition of VBNC bacteria is indeed different from that of logarithmic phase cells
- ❑ The  $K^+$  in the cytoplasm leaks

**Decreased**

Membrane  
potential

Unsaturated  
fatty acid

Membrane  
fluidity  
levels



# Physiology

- ❑ Transmission efficiency of nutrients and substances is lower
- ❑ The metabolism and respiratory activity decreases
- ❑ Gene expression levels are altered

High levels of rRNA

Undamaged genetic information

Plasmid

High levels of ATP

High levels of cAMP

Aggregation of proteins

# Virulence

Bacterial virulence decreases after entering the VBNC state

*Aeromonas hydrophila*

Enterotoxigenic *E. coli*

*H. pylori*

*E. coli* O157:H7

Some VBNC bacteria still retain basic virulence

VBNC *Legionella*

*Campylobacter jejuni*

*Listeria monocytogenes*  
and *Salmonella enterica*



Vora et al.

used a microarray study  
with 95 oligonucleotides

*Vibrio* species

Express genes

Toxins

(*ctxAB*, *rtxA*, *hlyA*, *tl*, *tdh*, *vvhA*)

Virulence  
factors

(*tcpA* and *TTSS*)

Lothigius et al.

VBNC cells of  
*E. coli*

*estA* (heat-stable toxin)

*eltB* (heat-labile enterotoxin)

*cfaB* (colonization factor )

*csvA* (fimbriae major subunit)

*cvbB* (fimbriae periplasmic chaperone)

# Detection of viable but nonculturable bacteria



# Acridine orange staining

- After the addition of nalidixic acid

Purpose of reaction : Distinguishing actively growing cells from VBNC or dormant cells

Actively growing cells

Become elongated and fluoresce reddish orange

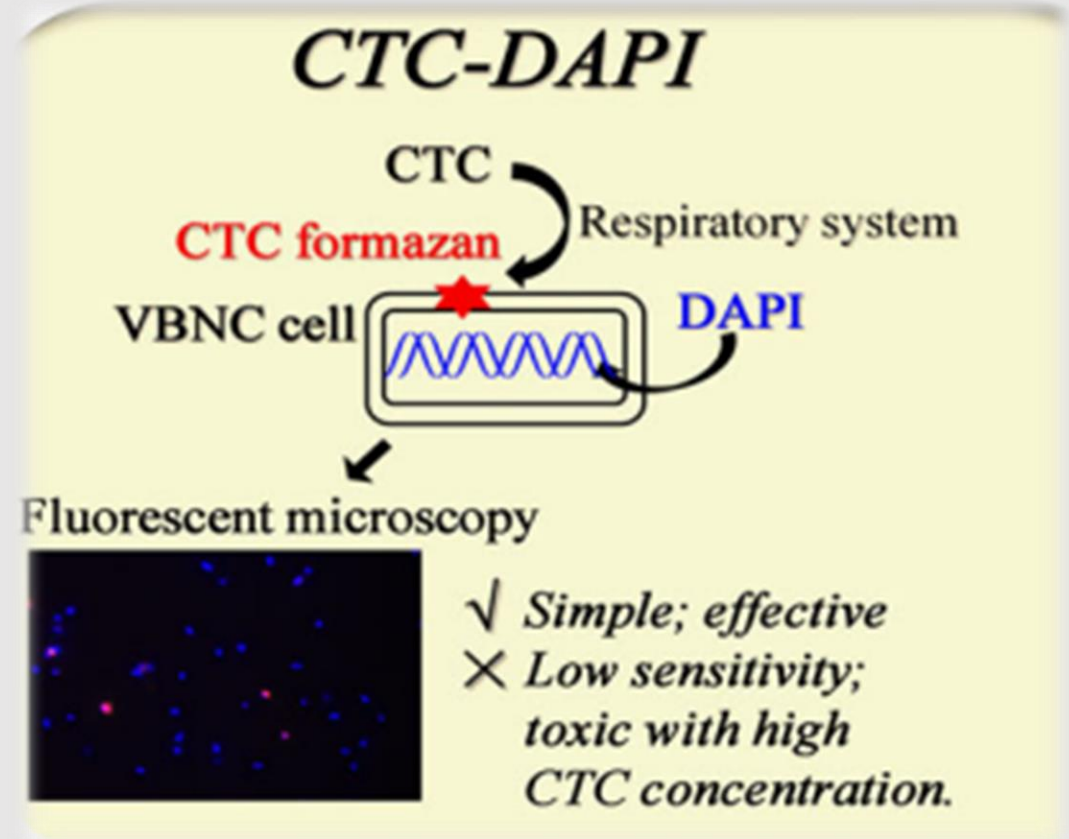
VBNC or dormant cells

Remain normal shaped and have greenish white fluorescence

Counterstaining with 5-cyano-2,3-ditolyltetrazolium chloride (CTC) and 40,6-diamidino-2phenylindole (DAPI)

Dead cells:  
blue

Viable cells :  
Red



# Live/dead baclight assay

A common method to detect total viable cells



Standard method

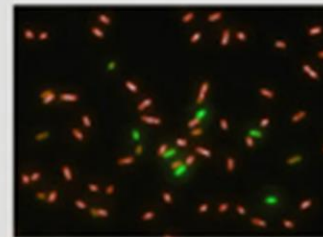


Positive and negative controls

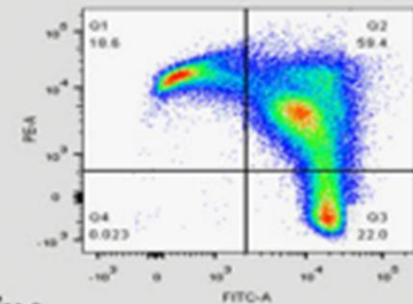
## Live/Dead BacLight staining



Fluorescent Microscopy



Flow Cytometry



✓ *Accurate, rapid results, sensitive.*

✗ *Intermediate states are generally misclassified and poorly characterized, not specific.*

# QPCR-based assays

- ❖ Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

↓  
mRNA

- ❖ Real-time (quantitative) polymerase chain reaction (qPCR)

↓  
Expose cells to specific compounds that penetrate membrane-permeable cells



Damage to nucleic acids

Inhibiting PCR amplification of non-viable cells



# Induction conditions



# General induction conditions

High/Low  
temperature

Disinfectants

Light-based  
disinfection  
technologies

Acidity/  
Alkalinity

Antibiotics

Co-cultivation

lactic acid bacteria

*L.monocytogenes*  
or  
*S. enterica*

VBNC

# Induction by light-based disinfection technologies

Distribution systems

Wastewater treatment plants (WWTPs)

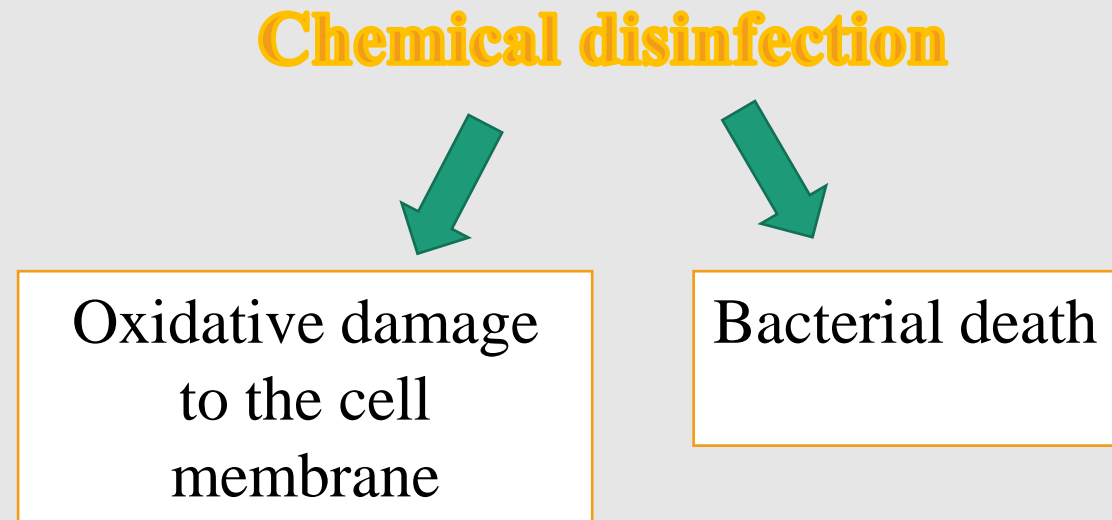
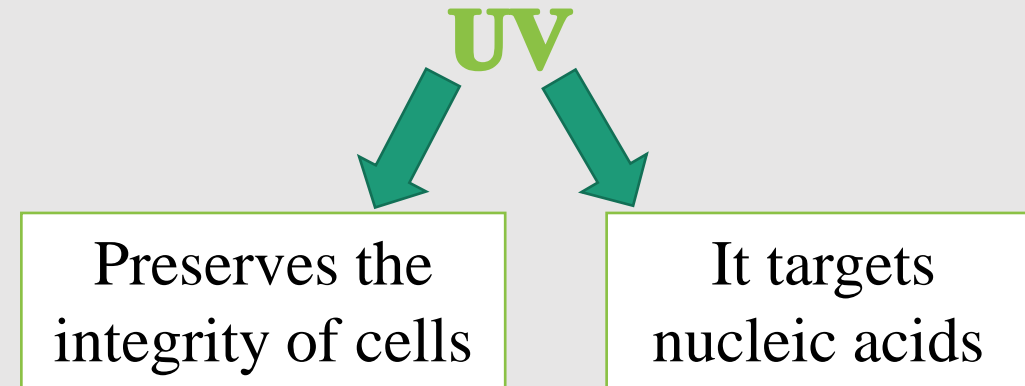
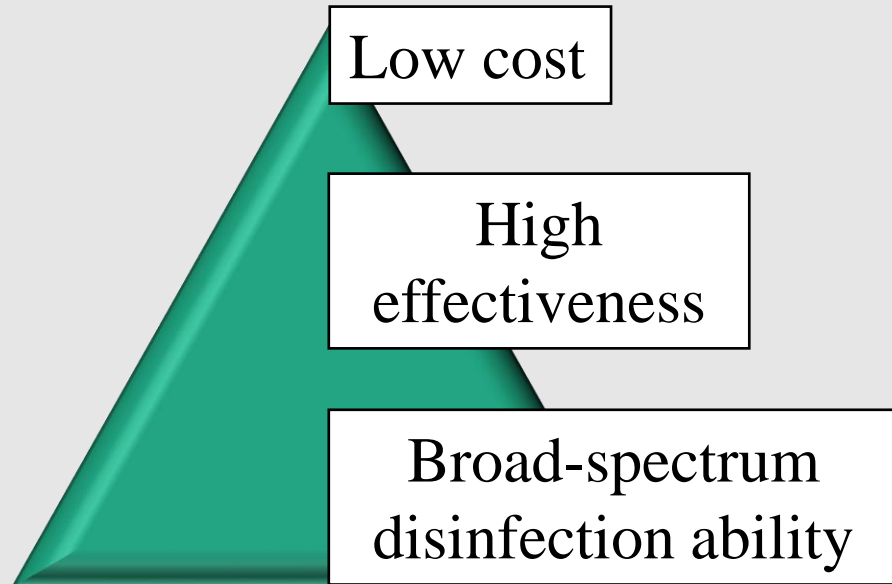
Disinfection is an essential and final process to ensure the safety of water

purpose

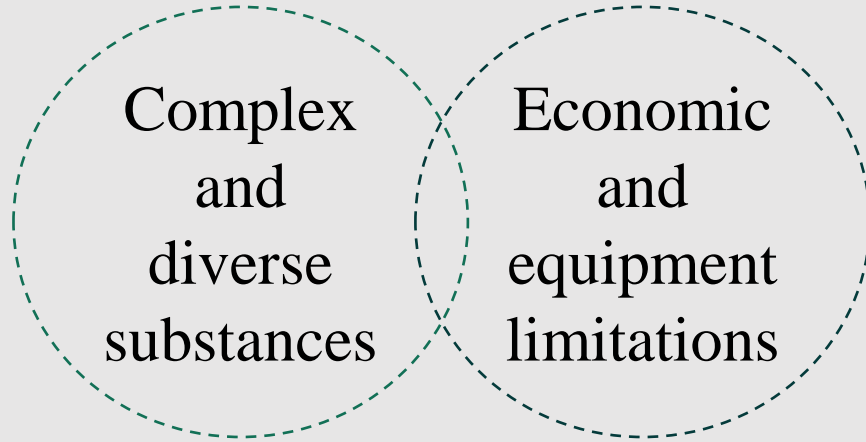
kill pathogenic microorganisms in the water

# Ultraviolet irradiation

- UV is the most common light-based disinfection technology used in water disinfection processes

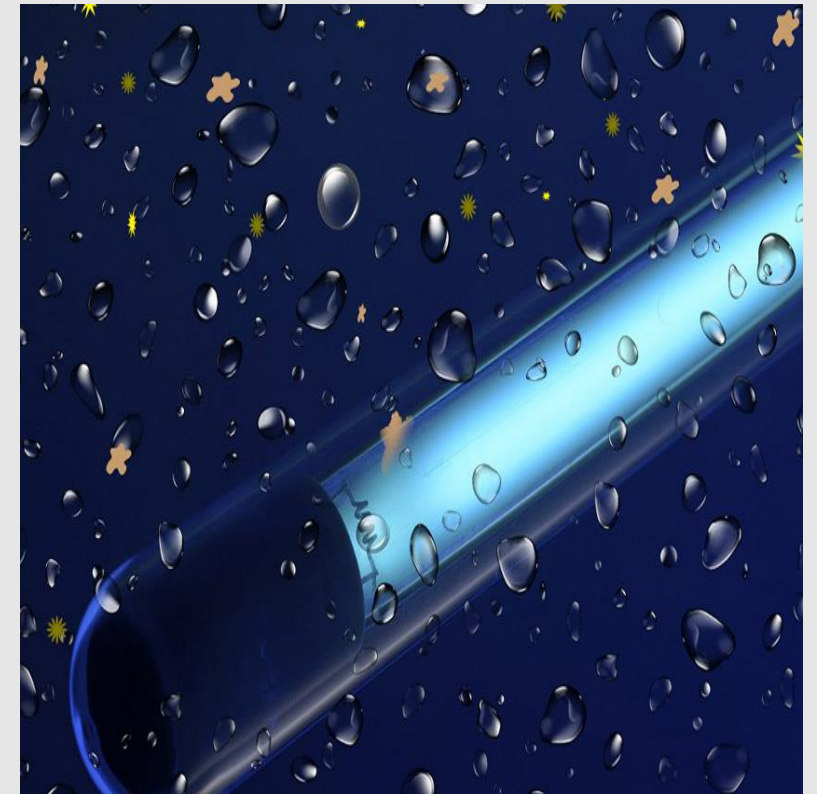


Conventional UV doses cannot guarantee complete inactivation of bacteria:

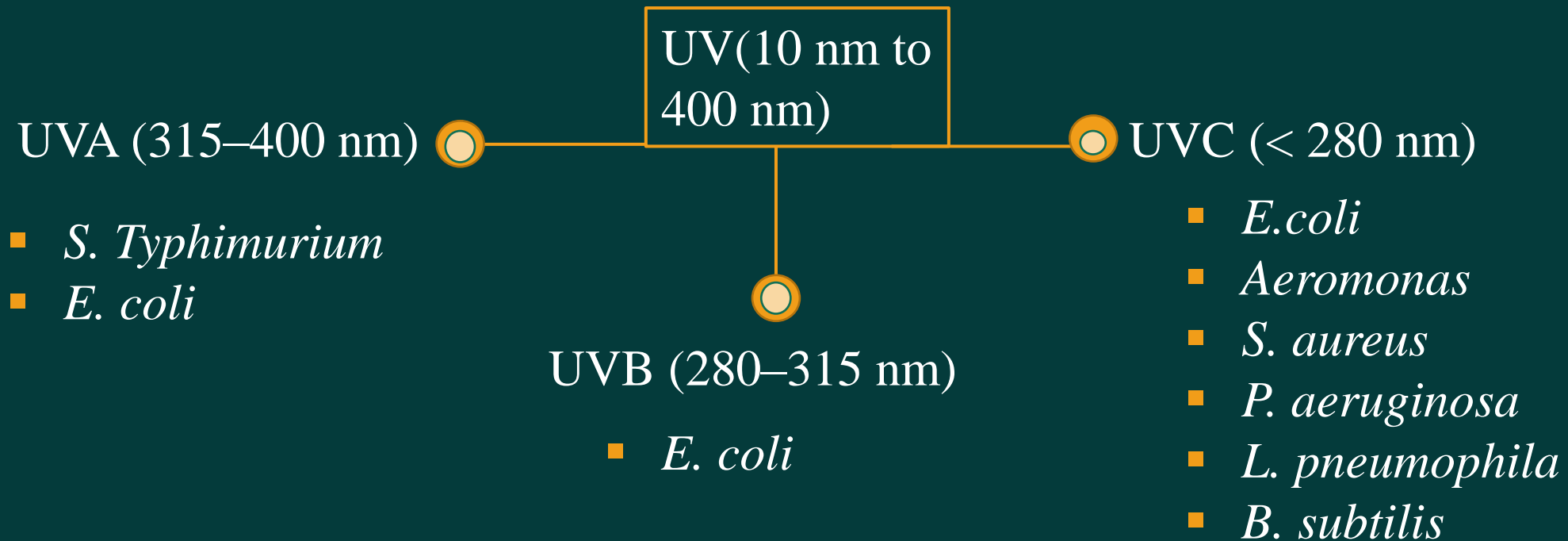


Biofilm could form and cover the surface of the UV lamp

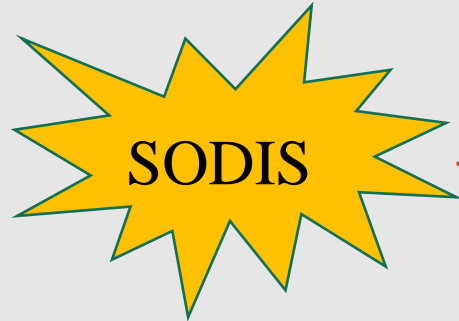
- Hindering the penetration and intensity of the UV
- Thereby reducing the disinfection efficiency



□ UV has no lasting residual disinfection ability



# Sunlight and Visible light



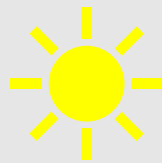
Use UV and thermal heating

Saltwater containing *Salmonella* and *E. coli* to natural sunlight



*Salmonella* entered the VBNC state

Seawater contains a large amount of NaCl



Cl<sup>-</sup>

Produces OH

The oxidation attack of OH leads to the formation of VBNC bacteria

# Pulsed light and gamma rays

- ❖ Pulsed light and gamma rays are also widely used as disinfection technologies for food safety

One or several high-power ultra-short-duration broad-spectrum (200 -1100 nm)

*Listeria innocua* and *E. coli*

Gamma rays are similar to visible light, but with much higher energy

DNA damage

- ❖ *L. monocytogenes*
- ❖ *S. aureus*
- ❖ *E. coli* O157:H7



# Photocatalysis

- Photocatalysis is a process in which a material absorbs light and triggers chemical reactions that break down pollutants and kill microorganisms.

UVA and TiO<sub>2</sub> treatment

- ❖ *E. coli*
- ❖ *S. aureus*
- ❖ *P. aeruginosa*

UVA, UVC and simulated sunlight with the catalyst TiO<sub>2</sub>

- ❖ *E. faecalis*

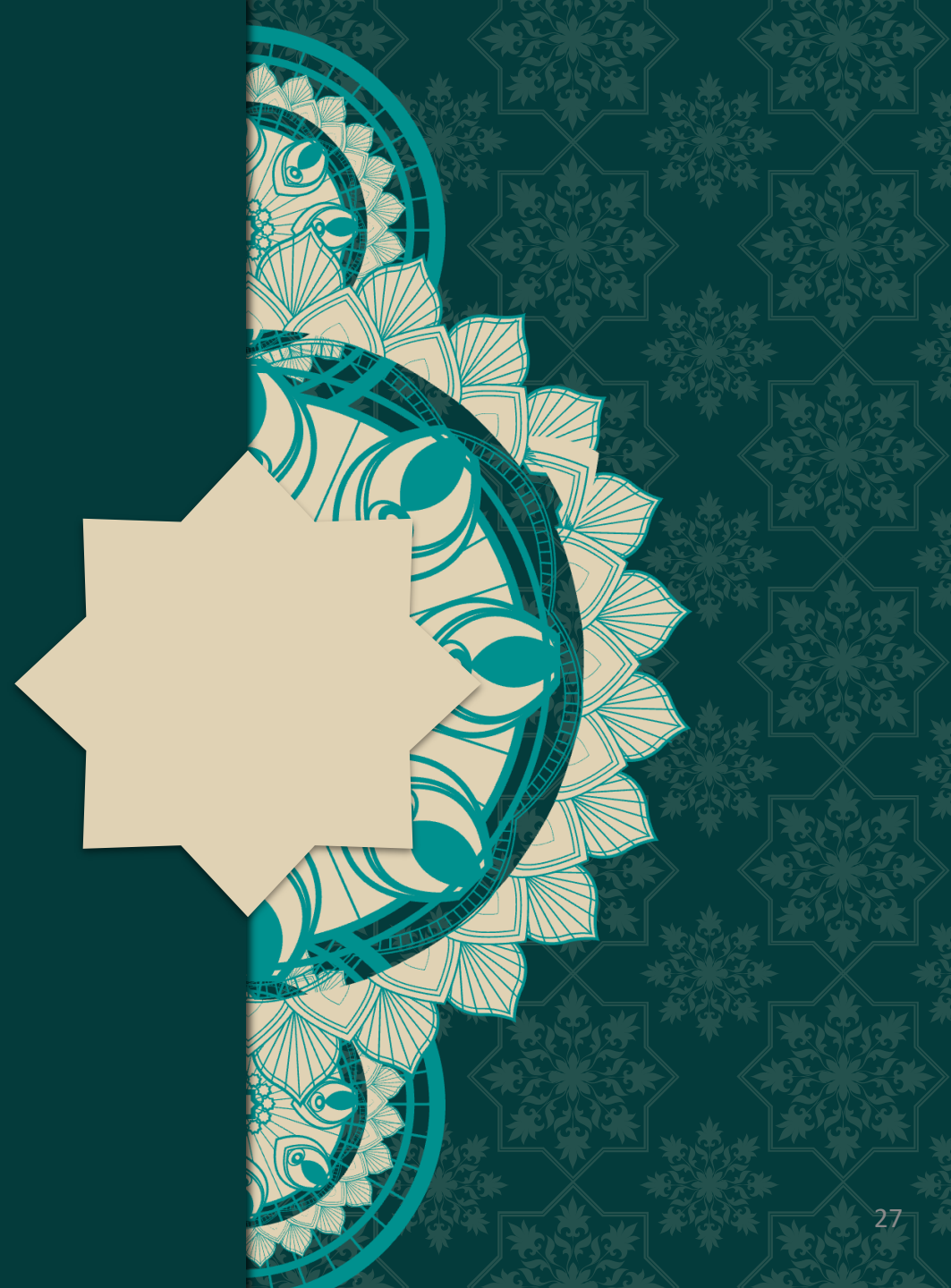
Alternative technology

Photo-oxidation  
disinfection

H<sub>2</sub>O<sub>2</sub> + simulated sunlight irradiation

- ❖ *E. coli*
- ❖ *K. pneumoniae*

# Formation mechanisms



Two major hypotheses

“Cell recession theory”

Oxidative damage

“Gene regulation theory”

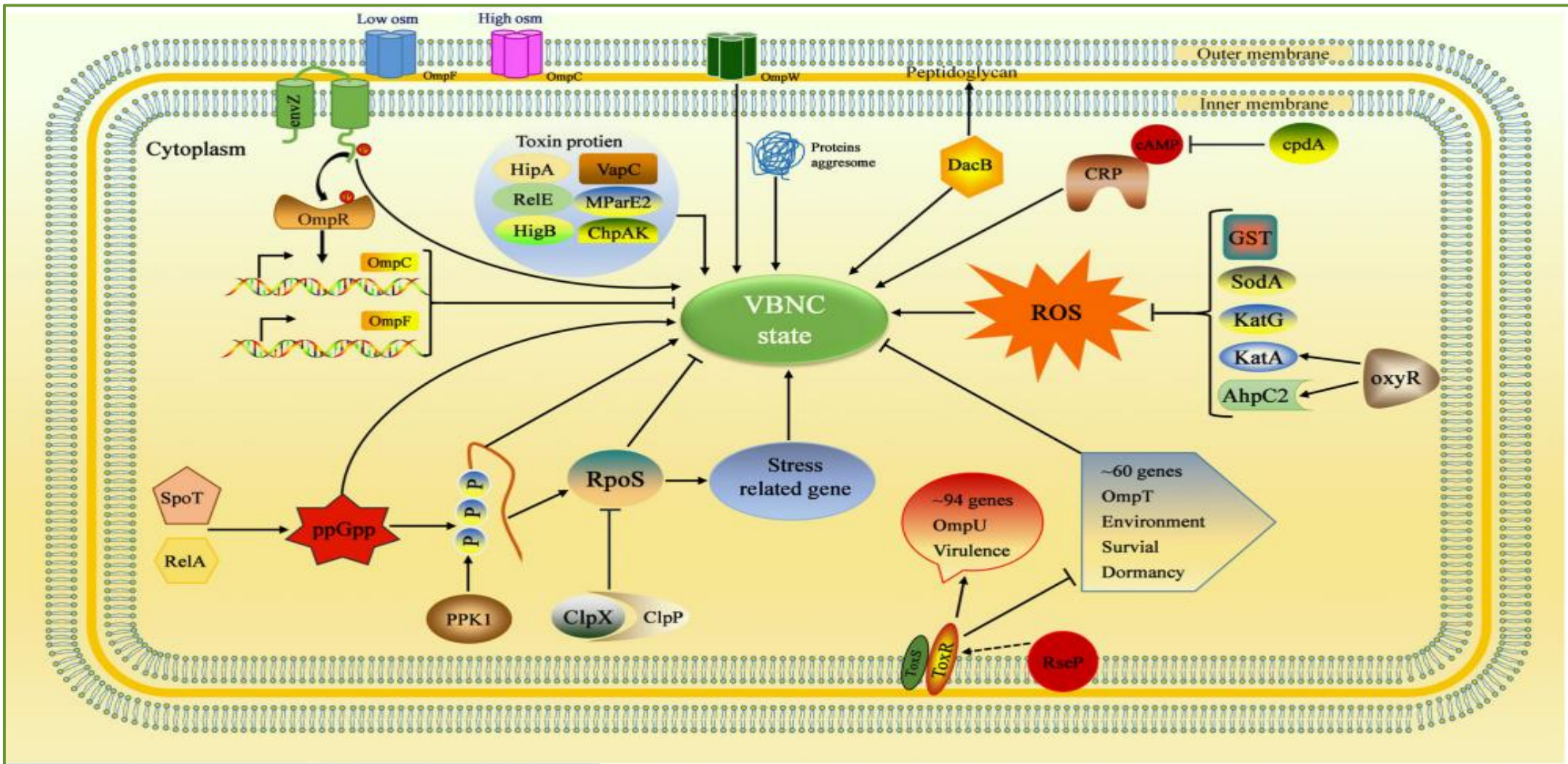
Gene regulation

Mechanisms

Stringent response

General stress response(RpoS),(oxyR)

Toxin-antitoxin system



(p)ppGpp: Guanosine tetraphosphate and guanosine pentaphosphate  
 PPK: Polyphosphate Kinase  
 ClpX : Caseinolytic Protease X  
 RpoS : RNA polymerase sigma S  
 ROS: Reactive oxygen species

# Toxin-antitoxin (TA) system

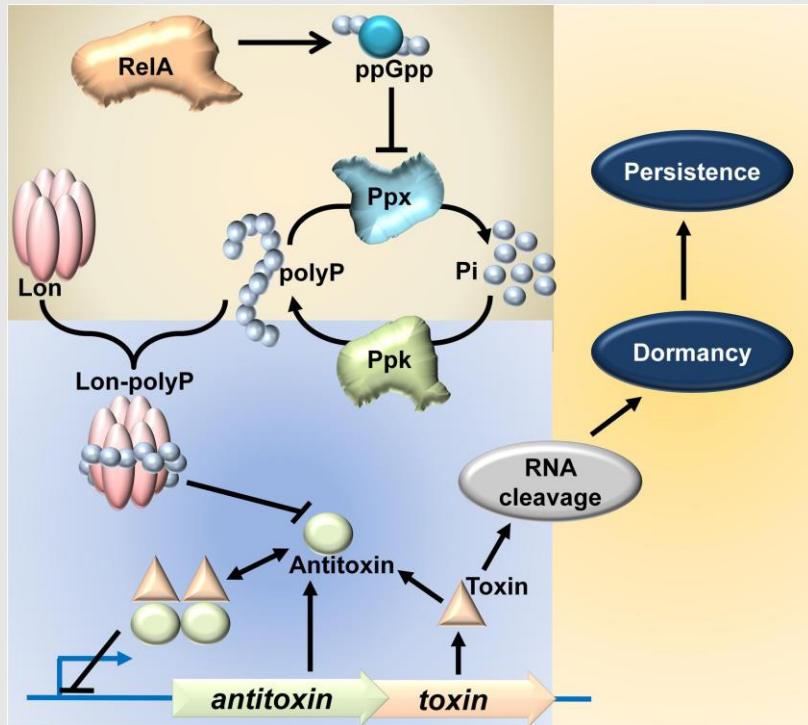
Consists of two co-expressed genes

One gene encodes a stable toxin protein

Enzymes

The other encodes an unstable antitoxin

RNA or proteins



Lon protease

Degradation of antitoxins

The toxins become free and active, blocking the main cell activities

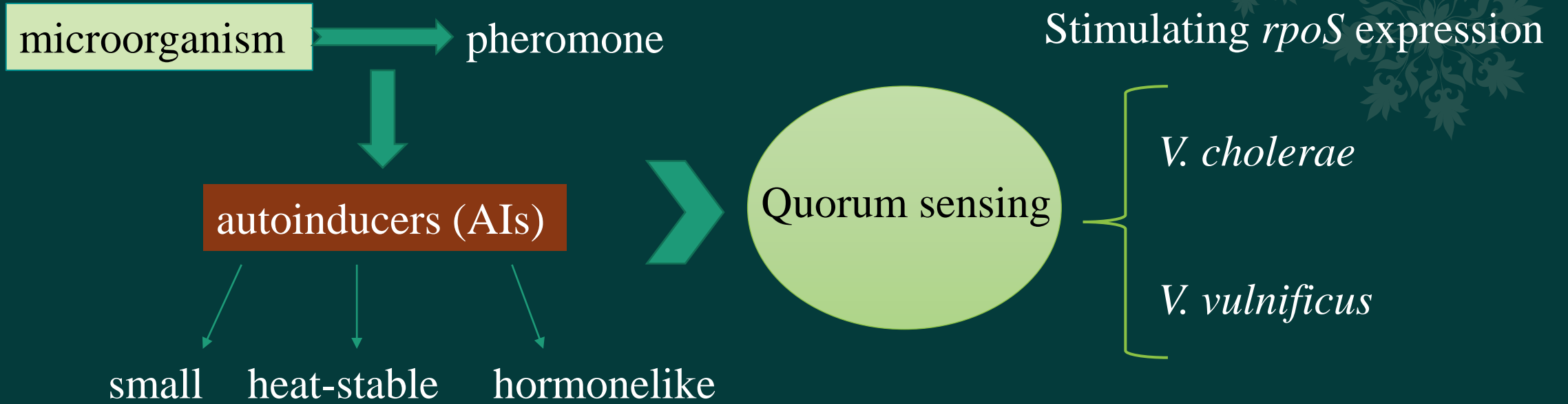
# Bacterial resuscitation



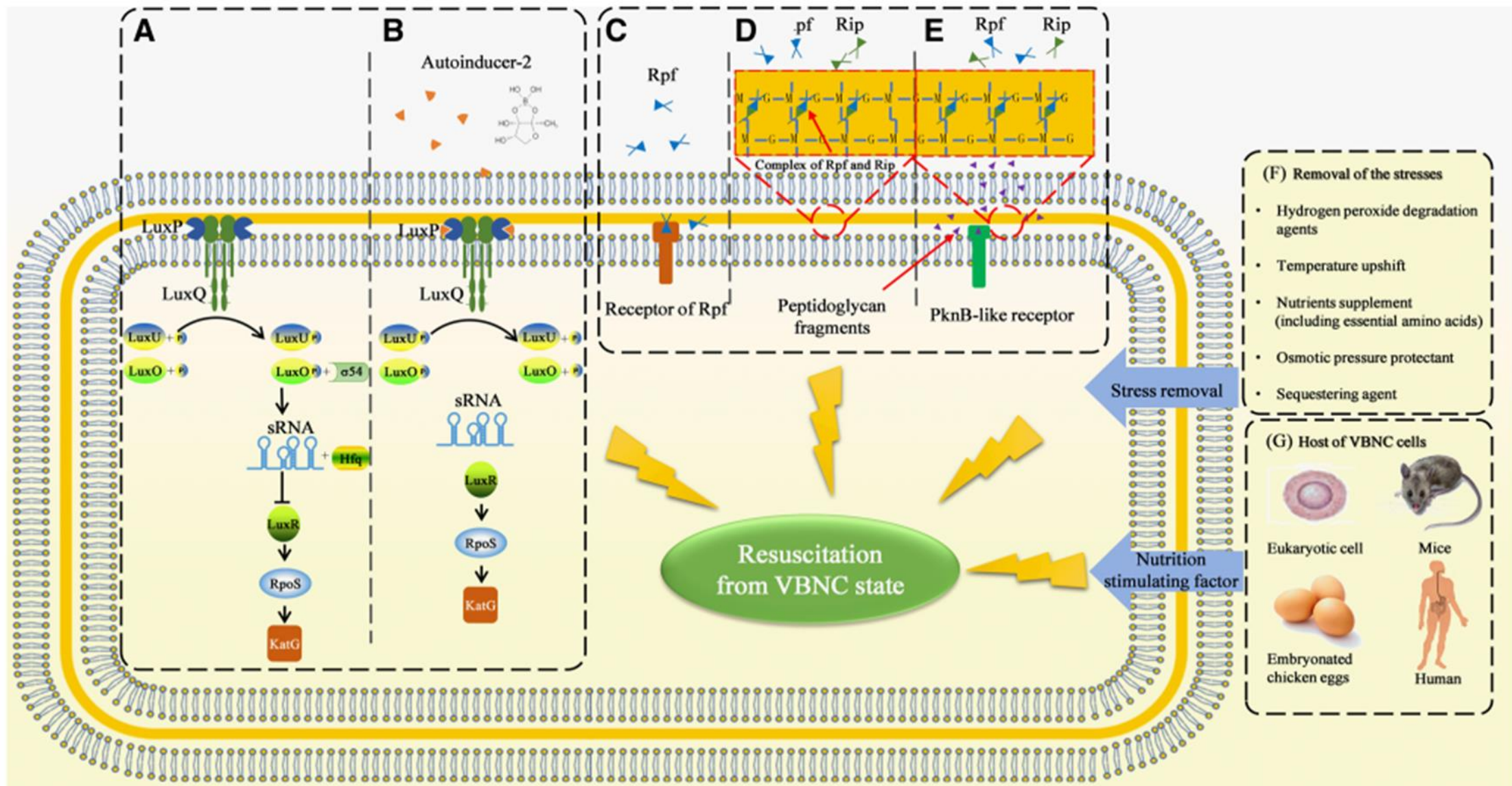
- ❑ Resuscitation is defined as the process by which VBNC cells regain culturability, which implies that the physiological and metabolic processes of the cells return to normal levels



# Autoinducers







# Resuscitation promoting factors

❖ (Rpf) → cytokine-like extracellular protein → Gene →

Gram positive bacteria with high DNA G + C content

❖ *M. luteus*

❖ *rpf* gene homologues

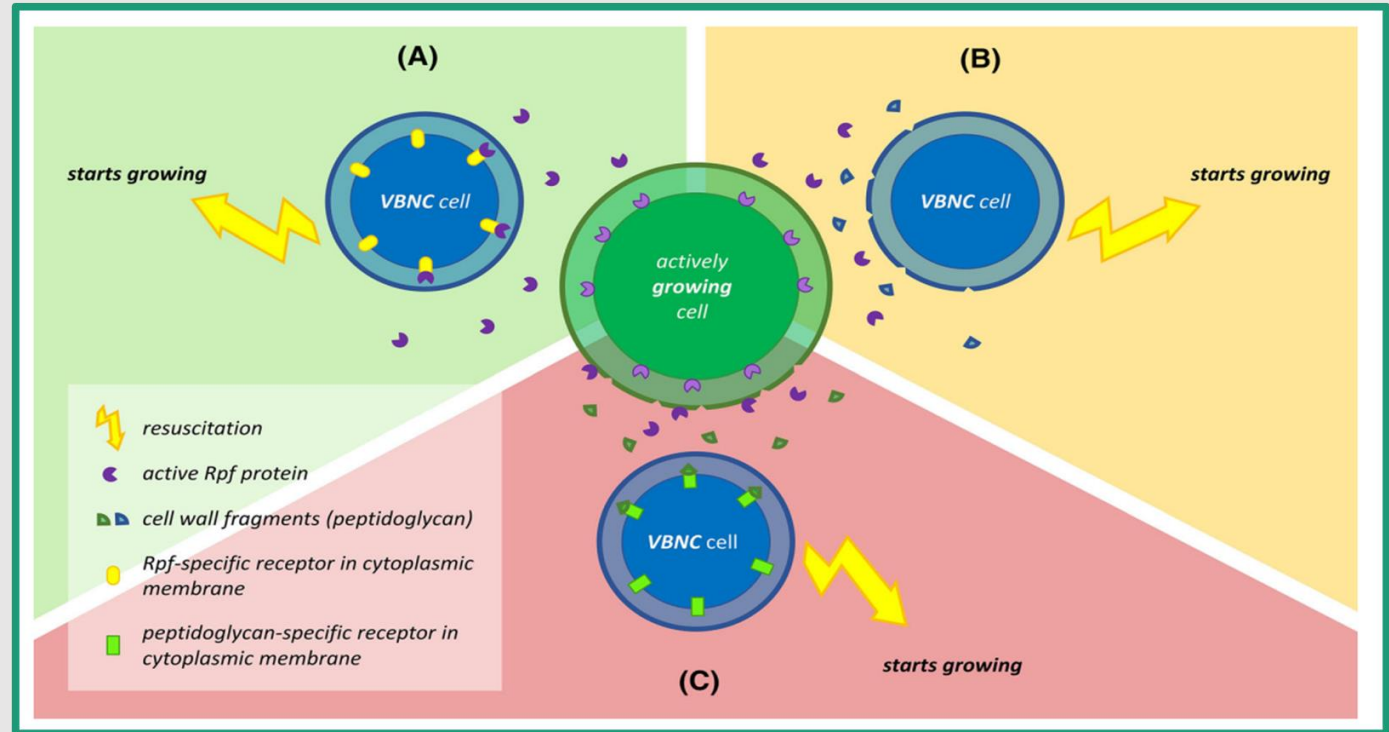
*Mycobacterium tuberculosis*

*M. Leprae*

*M. smegmatis*

*Corynebacterium glutamicum*

*Streptomyces specie*



# Siderophores

- ❖ Siderophores are small molecules produced by microorganisms to chelate ferric ions ( $\text{Fe}^{3+}$ )

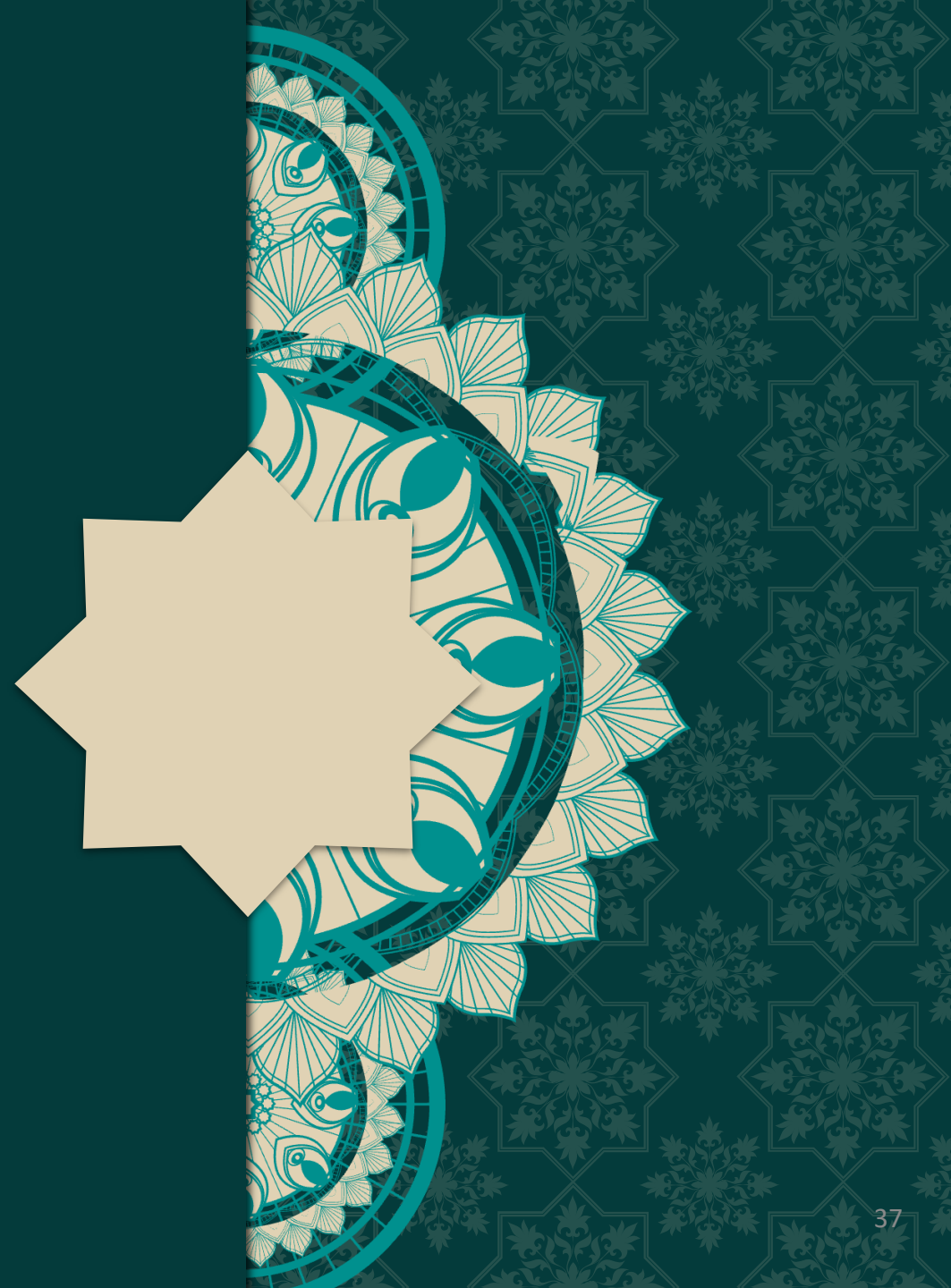
Growth  
Factors

Certain siderophores, act as growth factors by reducing the lag phase in bacteria like *Bacillus megaterium*, thus promoting cell division

siderophore piracy

Black queen hypothesis

# Conclusion



## VBNC state

resist various stresses, such as pasteurization, antibiotics, acids, and oxidation.

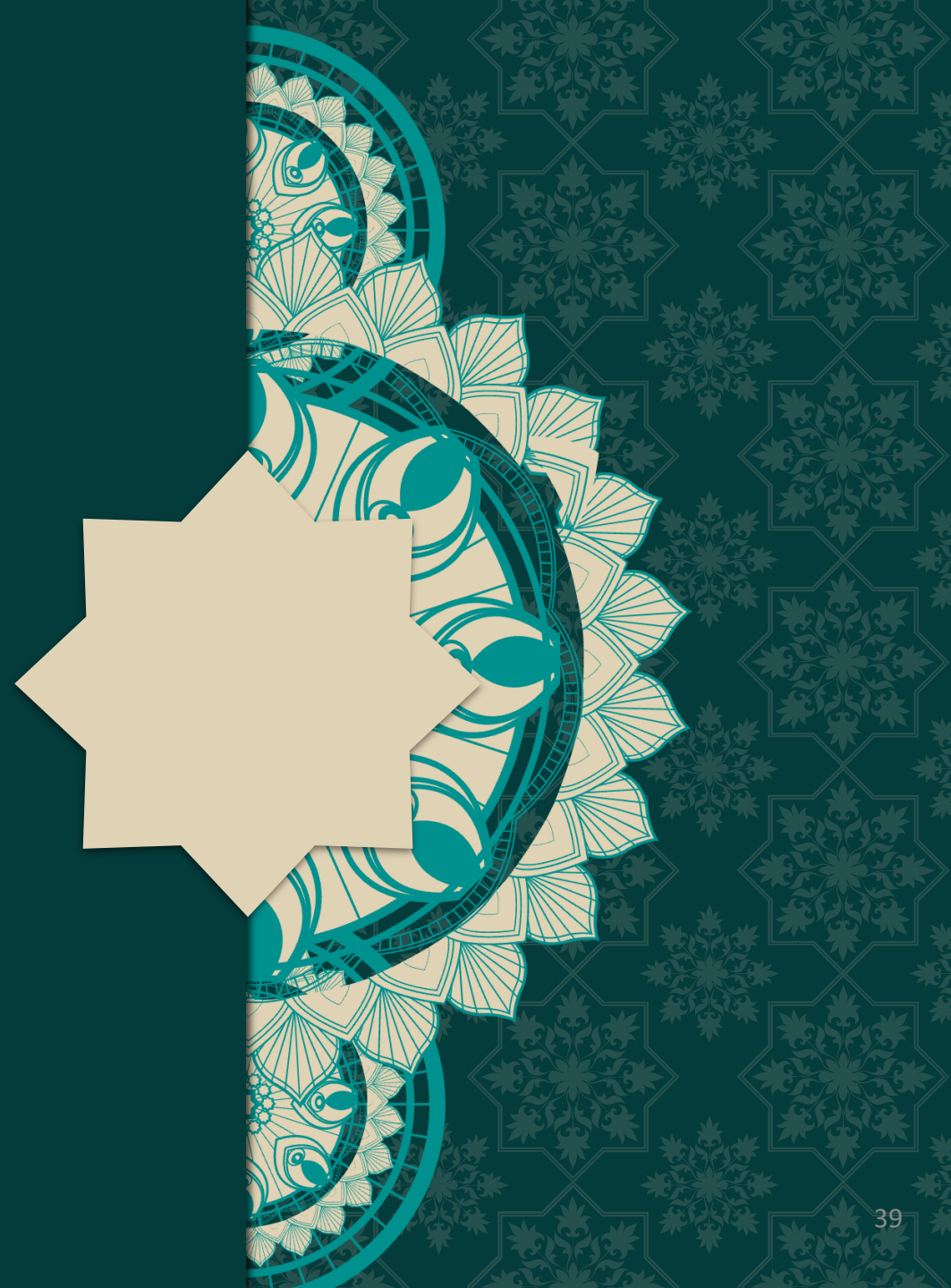
- ❑ May retain pathogenicity or regain virulence after resuscitation
- ❑ Many studies related to the VBNC state are based on a mixed system, and injured cells , culturable cells, and dead cells

## detection method

fast, sensitive, inexpensive, easy

- ❑ The universal mechanisms of VBNC state induction or resuscitation should be elucidated

# References



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