



Bacteriophages: Ecological role in the marine environment and potential applications

Sahar W. M. Hassan, Hanan Abd-ElNaby, Gehan M. Abou-Elela,
Ahmed E. Abouelwafa, Nermeen A. El-Sersy *

Marine Microbiology Lab., Environmental Division, National Institute of Oceanography and Fisheries (NIOF), Egypt.

*Corresponding author: nermeen_ok@yahoo.co.uk

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ABSTRACT

Bacteriophages are the most plentiful organisms on the earth and they are a ubiquitous trait of prokaryotic existence. They exist in all environments as a part of a complex microbial ecosystem. A phage is a virus that can infect bacteria where the distribution and abundance are based on the host organisms. Bacteriophages can impact their host populations in different ways and also have impacts on the microbial ecology and geochemical cycles. The concept of phage therapy as a fundamental application of bacteriophages is deeply relying on the facts of their host specificity, abundance in the environment with limited ecological impact. This review focuses on the ecological role of bacteriophages in the marine environment and some applications.

INTRODUCTION

Bacteriophages (phages) are bacteria-specific viruses. They are the most abundant biological entities on earth (**Katsamba and Lauga, 2019**). They exist in all ecosystems. The microbiota of healthy humans also contains a large number of bacterial viruses (**Van Belleghem *et al.*, 2019**). However, presentation of their genomic sequences in public databases is still limited. It was reported that only 1956 bacteriophage genomes are currently available (**Kazimierczak *et al.*, 2019**).

The ecological role of bacteriophages has an effect on bacterial existence, diversity in the ocean, biogeochemical cycles, regulation of nutrients and they are the key players of horizontal gene transfer and control of bacterial population size (**Breitbart *et al.*, 2018**).

Antimicrobial resistance is a main public health problem that can possibly cause an expected 10 million mortalities per year by 2050 (**Sugden *et al.*, 2016**). The crisis of antimicrobial resistance has been attributed to the absence of new agents or misuse of

these agents. Inclusive efforts are needed to reduce the pace of resistance and find suitable alternatives (**Baloch *et al.*, 2018**).

Recently, bacteriophages have been proposed as alternatives to antibiotics for many antibiotic resistant strains and have been applied in different fields of medical science and biotechnology including rapid detection and control of bacterial diseases (**Oechslin, 2018**).

Regarding this concept, the use of phage therapy in aquaculture seems to be very promising, the most feasible and suitable candidate to overcome the problems of antibiotic resistance. Bacteriophages are believed to protect fish against different pathogens and decrease the side-effects for the humans and environment. In aquaculture, addition of bacteriophages to live feeds, such as rotifers or *Artemia*, is an efficient method to purify the feeds before the entry to invertebrate or fish and provides less risk of bacterial resistance. In addition, they can be added in rearing tanks, which permit for co-evolution of phage-bacteria.

History of bacteriophages

The history of bacteriophage discovery started when Ernest Hankin, a British bacteriologist, reported in 1896 the presence of antibacterial activity against *Vibrio cholerae* in the waters of the Ganges and Jumna rivers in India, caused by unidentified source (which passed through fine porcelain filters and was heat labile) and was responsible for the elimination of cholera epidemics (**Kirby, 2012**).

Two years later, the Russian bacteriologist Gamaleya observed a similar phenomenon while working with *Bacillus subtilis* (**Marza *et al.*, 2006**). The rediscovery of bacteriophages by d'Herelle is frequently associated with an outbreak of severe hemorrhagic dysentery among several soldiers and he was assigned to make a realization for this incident.

Structure of bacteriophages

The classical structure of bacteriophage is composed of head and tail (Fig. 1). The head is a coat of protein and has an icosahedron shape and comprise the genetic material of the phage. The tail exhibits six tail fibers with receptors to recognize specific sites on the bacterial surface. It was stated that the morphology and nature of each bacteriophage determine the action used by the bacteriophage to inject its genome and also determine the style of its life cycle (**Mansour, 2018**).

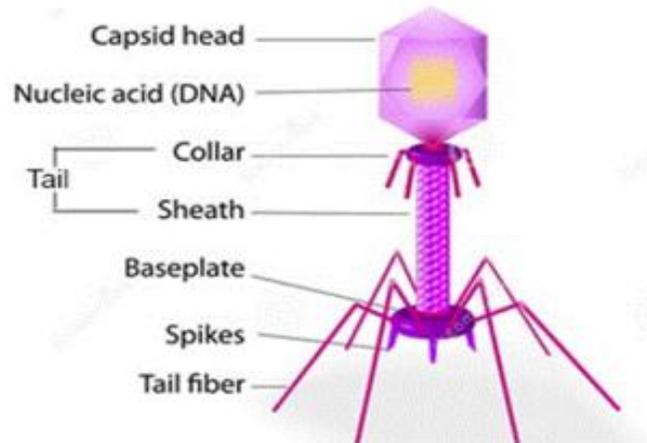


Fig1: Typical bacteriophage structure (Mansour, 2018).

Classification of bacteriophages

Eukarya, Bacteria and Archaea are the main domains of life and contain cellular organisms (Woese *et al.*, 1990). Bacteriophages cannot be incorporated into these domains due to lack of ribosomal RNA nucleotide sequence (Rohwer and Edwards, 2002), although sensitivity to infection with bacteriophages is a common feature in these domains.

Classification is mainly used to detect relationships among bacteriophages, to identify novel bacteriophage with industrial or therapeutic applications or harmful bacteriophages in fermentation industry for eradication and control purposes (Kutter and Sulakvelidze, 2005). Bacteriophages are classified into 13 families based on their virion morphology and the nature of their encapsidated nucleic acid (Hanlon, 2007). The three main families belonging to the Caudovirales are Siphoviridae, Myoviridae and Podoviridae (Kutter and Sulakvelidze, 2005; Lopes *et al.*, 2014).

The families with dsDNA are: Fuselloviridae (pleomorphic, envelope, lipids, no capsid), Lipothrixviridae (enveloped filaments, lipids), Corticoviridae (icosahedra capsid with lipid layer), Siphoviridae (long, non-contractile tail), Tectiviridae (icosahedral capsid with inner lipoprotein vesicle), Plasmaviridae (pleomorphic, envelope, lipids, no capsid), Podoviridae (short, non-contractile tail), Rudoviridae (helical rods), Myoviridae (contractile tail), Inoviridae (rod-shaped with helical symmetry) and Microviridae (icosahedral capsid) (Thung *et al.*, 2018) (Fig. 2).

The families with ssRNA and dsRNA are Leviviridae (quasi-icosahedral capsid) and Cystoviridae (enveloped, icosahedral capsid, lipids), respectively. It was reported that more than 96% of bacteriophages are tailed phages (Ackermann, 2007), belong to the order Caudovirales and exhibit varied properties, such as composition and content of

DNA, nature of constitute proteins, host range, serology, and physiology (**Kutter and Sulakvelidze, 2005**).

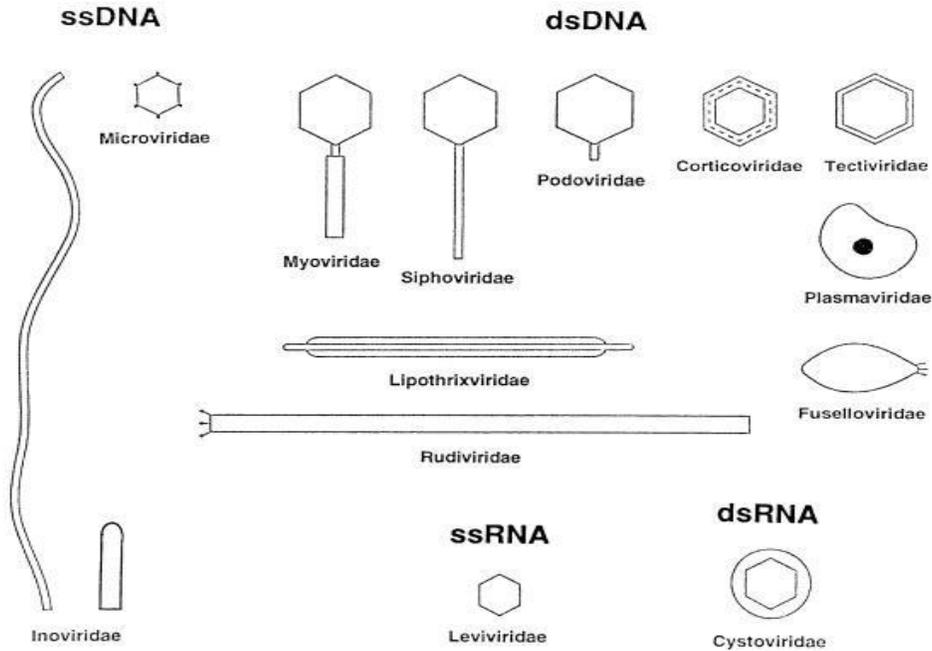


Fig. 2: Bacteriophages morphotypes (**Ackermann and Dubow, 1987**).

Life cycle of bacteriophages

Phages can infect bacterial cells and release by large number after infection and replication inside the host cells (**Sime-Ngando, 2014**). The main proceedings of infection are shown in Fig. 3. Phages are grouped as either temperate or virulent (lytic) according to the type of replication. In lytic cycle, once the cell has infected with a phage, the phage starts to direct the cell towards formation of new particles. Once the new phages have been formed, the lytic activity using some lytic enzymes including endolysins and holins occurs to release the new progeny (**Sime-Ngando, 2014**).

In case of lysogenic cycle, the temperate phage has the capability to integrate its DNA into the host genome to be replicated with the host genome and transmitted by daughter cells, which is called prophage. The prophage stays in dormant state until the induction of lytic cycle by the surrounding stress such as chemical agents and ultraviolet radiation. pseudolysogenic and chronic infections are also observed in the cycles (**Weinbauer, 2004**).

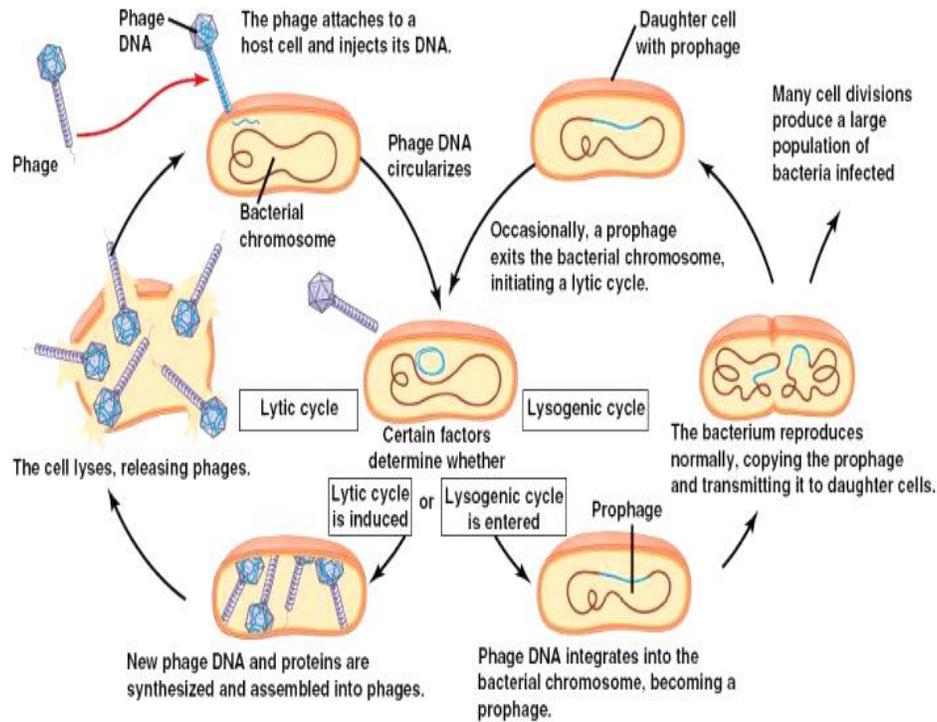


Fig. 3: Main steps of lytic and lysogenic phage infection cycles (Pal, 2015).

Lytic activity using bacteriolytic enzymes (Enzybiotics)

Bacteriolytic enzymes produced by bacteriophages are known as Enzybiotics. However, at present the word includes all enzymes with ability to kill bacterial pathogens (Keary *et al.*, 2013). Activity of Enzybiotics against Gram positive bacteria is illustrated in Fig. 4.

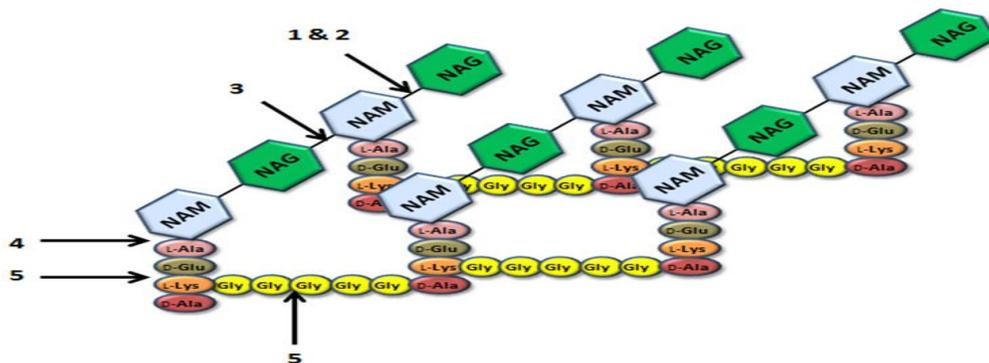


Fig. 4: The structure of *Staphylococcus aureus* peptidoglycan with the cleavage site of the five main types of enzymatic domain found in endolysins: 1) Nacetyl- β -D-muramidase (lysozymes), 2) lytic transglycosylase, 3) N-acetyl- β -D-glucosaminidase, 4) Nacetylmuramoyl-L-alanine amidases, 5) Endopeptidase. NAM: N-acetylmuramic acid. NAG: Nacetylglucosam (Keary *et al.*, 2013).

Bacteriophages in the marine environment

Most of the works are restricted to bacterial viruses of terrestrial origin. Marine bacteriophages have received only little attention (Sekar and Kandasamy, 2013). Bacterial viruses or bacteriophages in seawater were first observed in the first half of the last century (Kriss and Rukina, 1947). Phages have been reported to kill 20-40% of marine bacteria every day (Suttle, 2005). Hence, they are considered as the main player in ecological systems and bacterial evolution. They also have an important role in biogeochemical cycles (phosphorous, nitrogen and carbon cycles). Thus study of marine phages helped in understanding the regulation mechanism of aquatic systems (Sime-Ngando, 2014). Overall, studies concerning ecology of marine bacteriophages are roots of novel information about evolution and biodiversity of living organisms, and the functioning of ecosystem. Phage-host interaction in the marine environment has an important role on evolution and mortality of marine microbes (Garin-Fernandez *et al.*, 2018) (Fig. 5).

Abundance and distribution of bacteriophages in the marine environment

Phages are prevalent in all marine environments representing the highest abundant structure and are regarded as the greatest reservoir of diversity on the earth (Sime-Ngando, 2014), millions of phages exist in 1 ml of seawater (Bergh *et al.*, 1989). Different studies showed that the abundance of phages in the marine environment is approximately 10-fold more than bacteria and variation in the bacteria-phage interaction can be observed (Wigington *et al.*, 2016), also phage diversity may be high even at small scales (Flaviani *et al.*, 2017). It was reported that phage abundances in sediments is higher than that in the water column by 10–1000 times (Danovaro *et al.*, 2008).

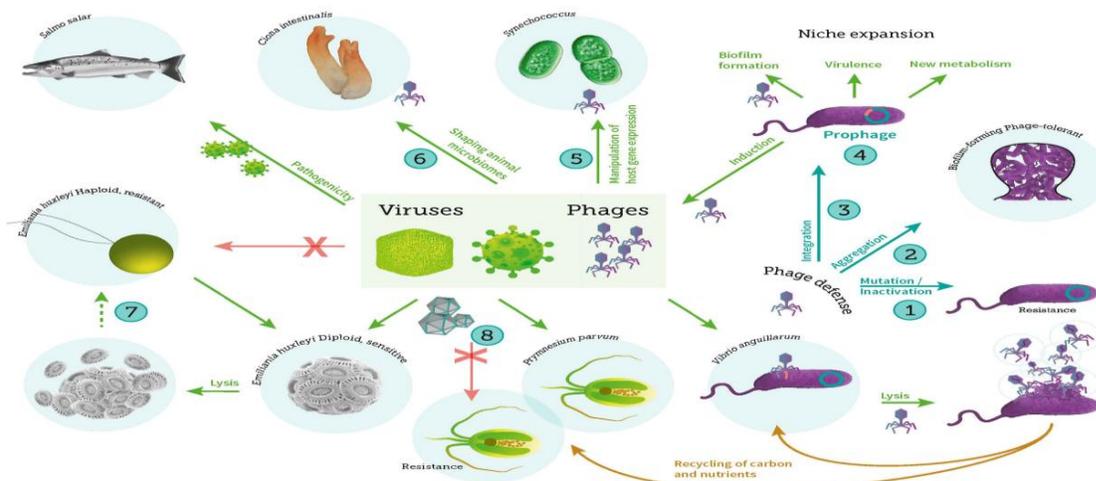


Fig. 5: Schematic overview of important phage-host interactions in the marine ecosystem (Middelboe and Brussaard, 2017).

Ecological role of bacteriophages in the marine environment

Effect of bacteriophages on microbial community and diversity

Mortality of bacterial cells by phages is the major factor controlling production and diversity of microbial community in marine systems. The majority of phages is not pathogens and has an effect on the release and reservation of organic matter in the pelagic universe. According to the fact of recombination between host genes and phage and also spread of phage-encoded biological functions, phage populations are important for storage of genes that participate to division of microbes in marine habitat. Moreover, they represent an important tool for evolution of genes in natural habitat, and could play an essential role in the genetic diversity of microbial populations (Sime-Ngando, 2014). Population of phages also contribute to classification of microbial species due to gene transfer by different mechanisms including transduction, transformation and conjugation (Fig. 6), which in turn affect gene evolution in the marine ecosystems (Sime-Ngando, 2014).

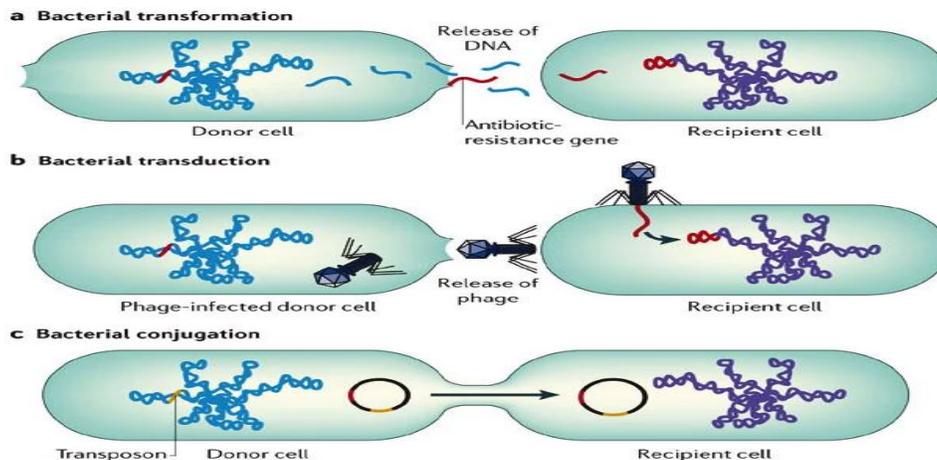


Fig. 6: Types of gene transfer: (a) bacterial transformation, (b) bacterial transduction and (c) bacterial conjugation (<https://vignette.wikia.nocookie.net>).

Transduction is a random action and has an effect on populations and transfer of genetic material in marine environment. It was suggested that about 1024 genes are transferred from phages to hosts by transduction each year (Rohwer and Thurber, 2009). Several helpful functional and metabolic genes were detected in the genomes of phage, representing a mutual benefit to phage and host that could be spread to other hosts through transfer of genes. These genes can participate to functional characteristics of the host such as virulence. They can also induce the formation of bacterial biofilm (Gödeke *et al.*, 2011).

An interesting example is the photosynthetic genes exist in the genomes of cyanophage, which can be used to preserve the function of photosynthesis in hosts, and can be moved between hosts (**Lindell *et al.*, 2005**). It was reported that genes originating from phages cause the achievement of about 10% of global photosynthesis (**Rohwer and Thurber, 2009**).

The release of host components after lysis by phages can alter the bioavailability and composition of organic substances and nutrients, which is considered as key player and affects the community structure and microbial composition. These levels are affected by host availability (density and activity), temperature, growth rate of the host cells, solar radiations and anoxia. This manner enhances the coexistence within communities or prevent dominance of species which can determine the diversity and community structure (**Pradeep Ram *et al.*, 2010**). Understanding of the interaction between host and phage is necessary to predict the effect of environment on phage-driven operations in sea. Important phage host interactions occur in the marine ecosystem are shown in Fig. 7.

Effect on biogeochemical cycling

Bacteriophages have great direct or indirect effects on the cycling of elements due to killing microbial hosts such as archaea, bacteria, cyanobacteria, which dominate in pelagic systems, and are the main partners in the food web. They have a great direct or indirect effect on cycling of carbon, nitrogen, phosphorus and other elements, which are essential in the food web (**Fuhrman, 1999**). It was reported that approximately 10^8 – 10^9 tons of carbon per day is released from the living biological aggregation and resulted from about 1029 viral infection events per day (**Suttle, 2007**). It was estimated that 25% of the carbon fixed through photosynthesis cycles is through the viral shunt (Fig. 8, 9). Thus lytic activity of phages is potent producer of nutrients to primary producers leading to increases of organic matter (DOM) retention time and respiration in the water column. A modeling exercise proposed that lysis of 50% of bacterial population caused 27% increase in microbial respiration (**Fuhrman, 1999**) and nutrient retention by decreasing of organic matter sedimentation (**Ankrah *et al.*, 2014**).

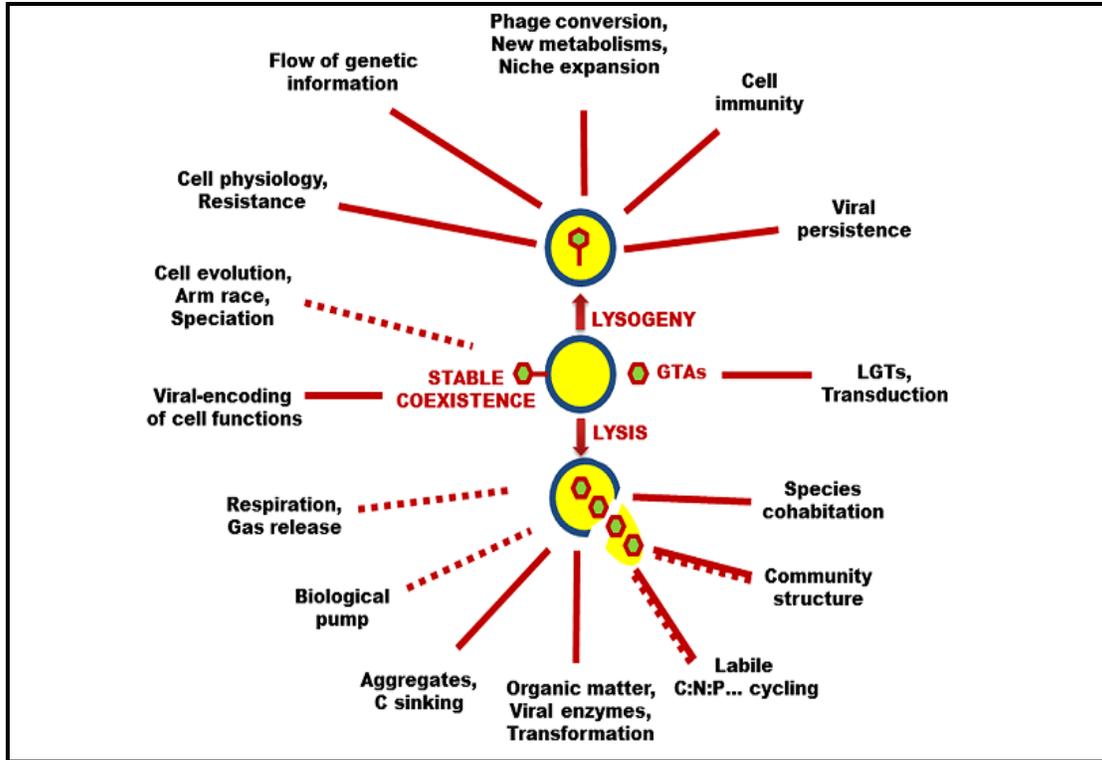


Fig. 7: Phage–microbe interactions (Sime- Ngando, 2014)

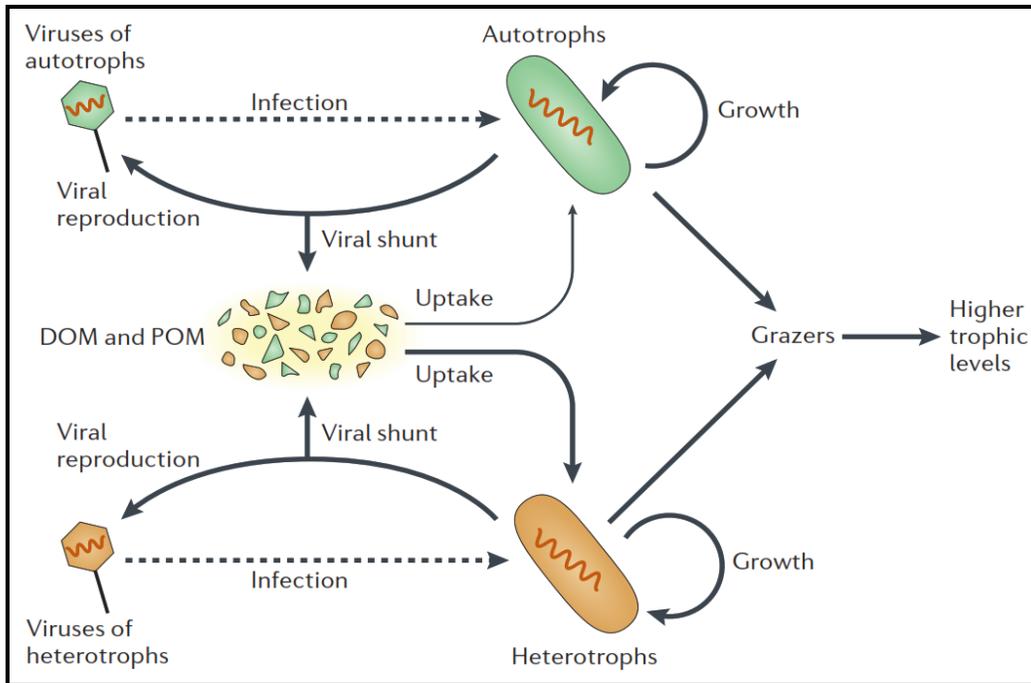


Fig. 8: Schematic of the viral shunt (Jover *et al.*, 2014).

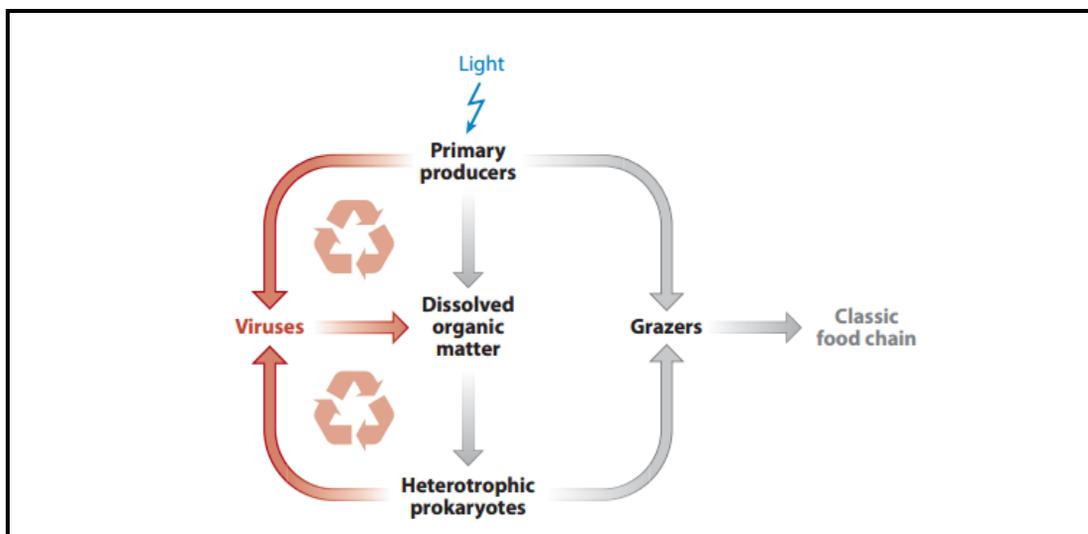


Fig. 9: Simple diagram of the marine microbial food web, with the viral shunt highlighted in red colour (Breitbart, 2012).

Some environmental applications of bacteriophages

1. Indication of faecal pollution in the marine environment

Disposal of wastewater in the marine environment causes serious health problems, because wastewater treatment methods remove only the suspended solids with some degree of pathogen elimination. The fast, easy, cost effective enumeration, detection of bacteriophages and the persistence of them in water in addition to their high concentration exist in areas contaminated with faecal remains encourage the use of them as indicators of water quality.

Dias *et al.* (2018) suggested the use of somatic phages as indicator of enteric viral pathogens than the current faecal coliform and that these phages will play a vital role in future health care planning.

2. Detection of pathogenic bacteria

The immobilized Landscape phage 1G40 was applied for detection of β -galactosidase from *E. coli* (Nanduri *et al.*, 2007). The immobilized filamentous phage on piezoelectric transducer was also used for the detection of *Salmonella typhimurium* (Olsen *et al.*, 2006).

Filamentous bacteriophage was used to detect *S. typhimurium* and spores of *Bacillus anthracis* in water and fat free milk (Lakshmanan *et al.*, 2007 a, b; Petrenko, 2008). It was observed that the frequency changes of resonance with time is related to mass of spores on the sensor. The captured spore cells were detected using scanning electron microscopy (SEM) (Fig. 11)

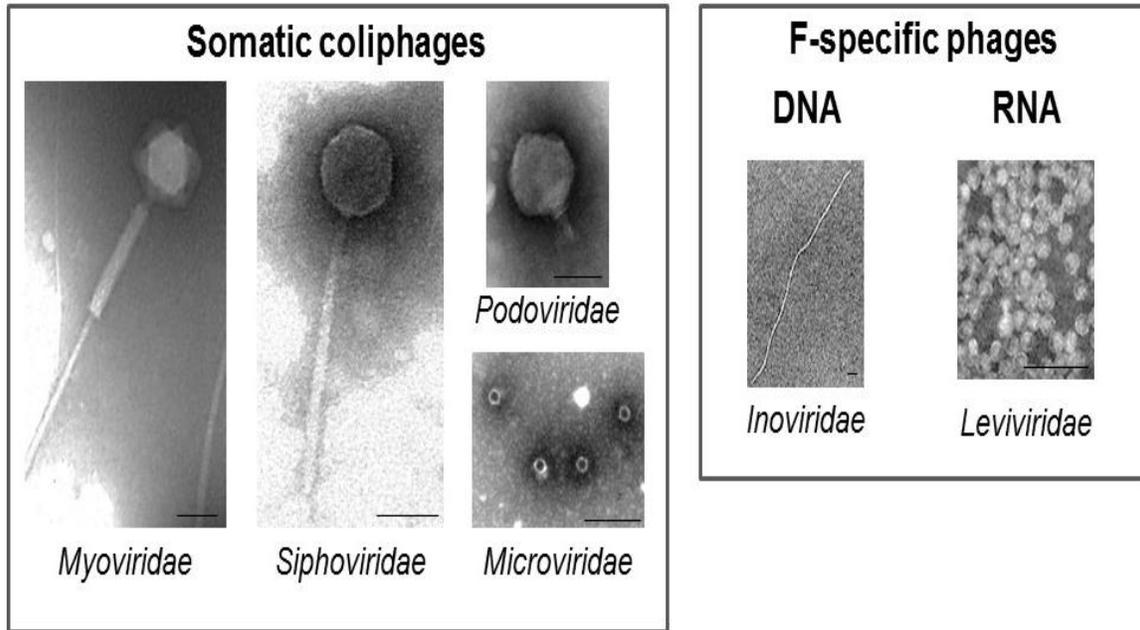


Fig. 10: The most common morphological types in somatic coliphages (a) and F-specific phages (b) (Jofre *et al.*, 2016).

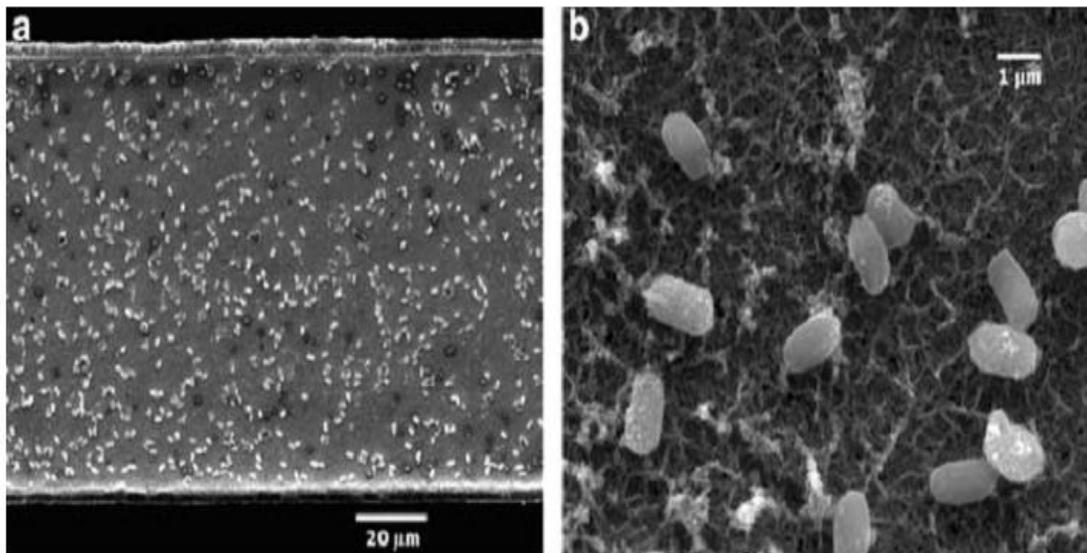


Fig. 11: SEM image of sensor coated with filamentous phage (a) and attachment of the spores to the phage (b). (Johnson *et al.*, 2008).

The platforms that have leveraged phage-based probes have used intact bacteriophage particles as the recognition element, and have effectively detected some pathogens in food (Fig. 12).

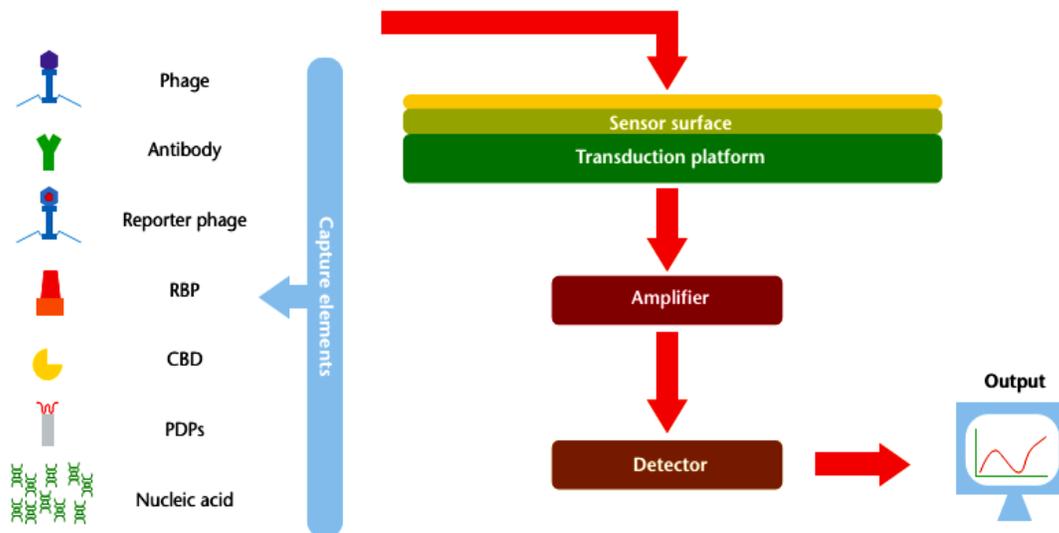


Fig. 12: The components of a biosensor showing various biorecognition elements that may be attached to the surface (Gutiérrez *et al.*, 2019).

3. Phage therapy in aquaculture

Phage therapy, the use of bacteriophage viruses to treat bacterial infections, has existed for more than a hundred years (Brives and Pourraz, 2020). Success of phage therapy against bacterial pathogens depends on different factors such as physico-chemical factors (pH, temperature...), the bacteriophage/bacteria ratio and resistance to phage or phage neutralization. The most effective parameters are the initial dose of phage, the burst size, latent period and phage adsorption rate (Payne and Jansen, 2001). Different phage products have been used in human medicine, agriculture, the food industry and has proven efficacy as a biocontrol agent towards aquaculture disease (Ninawe *et al.* 2020). A range of products have been approved by the Food and Drug Administration (FDA) (Ly-Chatain, 2014).

3.1. Biocontrol of common bacterial pathogens in aquaculture

3.1.1. *Vibrio* spp.

The fast development of industry over the the past decades led to occurrence of vibriosis in aquaculture (Kalatzis *et al.*, 2018). Clinical signs of vibriosis (Fig. 13) include hemorrhages on the skin, unusual swimming behavior, loss of appetite close to the water surface, increased mucus secretion, petechial hemorrhages in the muscle, anemia and intestinal necrosis. *Vibrio* spp. such as *V. parahaemolyticus*, *V. campbellii*

and *V. harveyi*, are recognized as the causative agents of luminous vibriosis in shrimp farm (Kalatzis *et al.*, 2018).



Fig. 13: Symptoms of vibriosis in cultured European seabass (Kalatzis *et al.*, 2018).

A potential of bacteriophages to control pathogenic *V. harveyi* in hatchery setting was reported by Kumar *et al.* (2006). Promising results have been shown in sea cucumber (Zhang *et al.*, 2016). Three phages (PVS-1, PVS-2 and PVS-3) were tested against *V. splendidus*. Different diets were prepared, where bacteriophages were added separately to each diet and another diet was prepared with a cocktail of the tested bacteriophages. Another set of diets supplemented with antibiotics were also prepared in addition to the un supplemented diets (control). The diets were given daily to the sea cucumbers for 60 days. Then the sea cucumber were immersed in seawater containing *V. splendidus* for two days, followed by monitoring the survival rates for ten days. The survival was 82% for diets containing cocktail of phages, 50%-65% for the diets supplemented with individual phages and only 18% for the control. In the same investigation, injection of healthy sea cucumbers with *V. splendidus* was carried out, while sterile sea water was used to inject the control group. Subsequent injection of the infected group with phage cocktail was done and the rates of survival were checked for ten days. It was noticed that the survival was 80% for the treated group, while it was only 20% in case of untreated group. Hassan (2017) isolated some vibriophages from seawater samples collected from Alexandria seashore, Egypt. Four phages namely P10, P16, P17 and P20 were chosen and tested for host specificity. It was reported that P17 exhibited broad spectrum of antibacterial activity and was stable at pH7. Phage host interaction was studied showing that P17 had burst size (100 PFU per cell) and latent period (10 min). The study also indicated the promising effect of P17 in control of *Vibrio* spp. invading aquaria of *Artemia salina*.

3.1.2. *Aeromonas* spp.

Aeromonas hydrophila is a motile rod, facultatively anaerobic Gram-negative bacteria. It causes hemorrhagic septicemia and fin and tail rot. The symptoms appear as lesions leading to loss of scales and fin erosion; abscesses and ulcers; gills and vents hemorrhaging. Silva *et al.* (2016) tested the efficiency of phage therapy to eliminate *A. salmonicida*, the causative of furunculosis, resulting in high morbidity and mortality of *Solea senegalensis* juveniles. They isolated a new phage and investigated its ability to

eliminate *A. salmonicida* in infected juveniles of *Solea senegalensis*. Results revealed the absence of mortality after 72 h in case of fish treated with phages.

Hassan *et al.* (2018) isolated four *Aeromonas* phages from seawater samples collected from Alexandria, Egypt. They studied the molecular characterization of them and optimization of culture conditions to improve the antibacterial activity of the most promising phage isolate (AP2) against *A. hydrophila*. The study also evaluated the potential of marine phages for therapy of motile *Aeromonas* septicemia caused by *A. hydrophila* in Nile tilapia (*Oreochromis niloticus*) showing 94% elimination of *A. hydrophila* in aquaria of Nile tilapia infected with *A. hydrophila* compared to aquaria without phage treatment. Morphological and histological examination of the tested liver tissues are shown in Fig. 14.

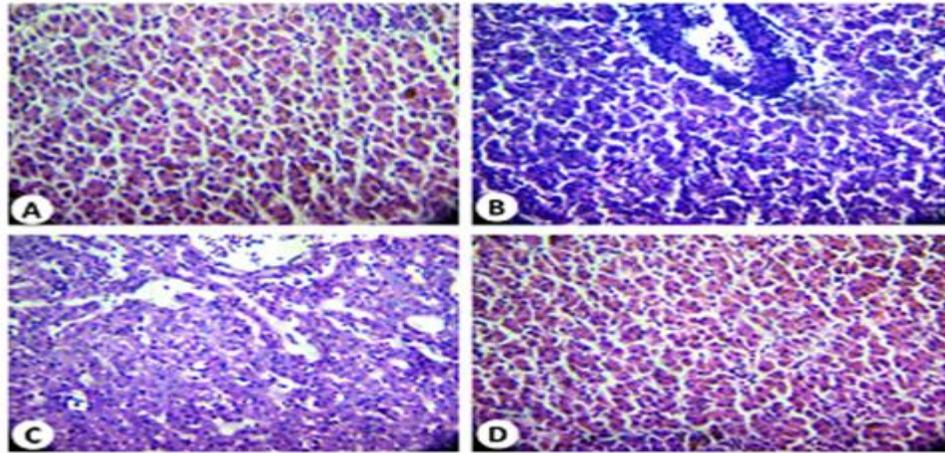


Fig. 14: Histopathological alterations of *Oreochromis niloticus* liver: (A): Normal liver tissue (control); (B): infected liver tissue showing disruption of the normal hepatic cord-like pattern, a great reduction in number and size of hepatocytes, (C): *O. niloticus* liver tissue treated with 1:1 (phage AP2: *A. hydrophila*) and (D) *O. niloticus* liver tissue treated with 1:2 (phage AP2: *A. hydrophila*) (D) (H&E: X, 400). **Hassan *et al.* (2018)**.

3.1.3. *Pseudomonas* spp.

Pseudomonas sp. causes hemorrhagic ascites in ayu fish (*Plecoglossus altivelis*). The clinical signs of infection appear as bloody ascites fluid and lesions in the spleen, liver, kidney, intestines, gills and heart (**Austin and Austin, 2012**). **Park *et al.* (2000)** reported the ability of isolated phages belonging to Podoviridae (designated PPpW-4) and Myoviridae (designated PPpW-3) to heal ulcerative lesions caused by *P. aeruginosa*. and the mortality reached 22.5% compared to 65.0% mortality.

3.1.4. *Streptococcus* spp.

Streptococcus sp. is a Gram-positive bacterium that causes streptococcosis (Figure 15) in fish and is responsible for 30–50% of the deaths in some fish. **Matsuoka et al. (2007)** carried out experiments on the Japanese flounder (*Paralichthys olivaceus*) by injection of the bacterial pathogen *Streptococcus iniae* and subsequent injection of phage isolates. They showed that less mortality of the fish was detected after 15 days.



Fig. 15: Symptoms of streptococcosis: exophthalmia hemorrhages around the gill plate, base of fins (**Tukmechi et al., 1999**).

3.1.5. *Edwardsiella* spp.

Edwardsiella ictaluri and *E. tarda* are the major species infecting fish. The infected fish suffers from lesions on their skin, loss of pigmentation, opacity of the eyes, swelling of the abdominal and mucus secretion (Fig. 16). **Hsu et al. (2000)** indicated the ability of the isolated phages to control *E.tarda* by about 1.5 log after 8h of treatment.

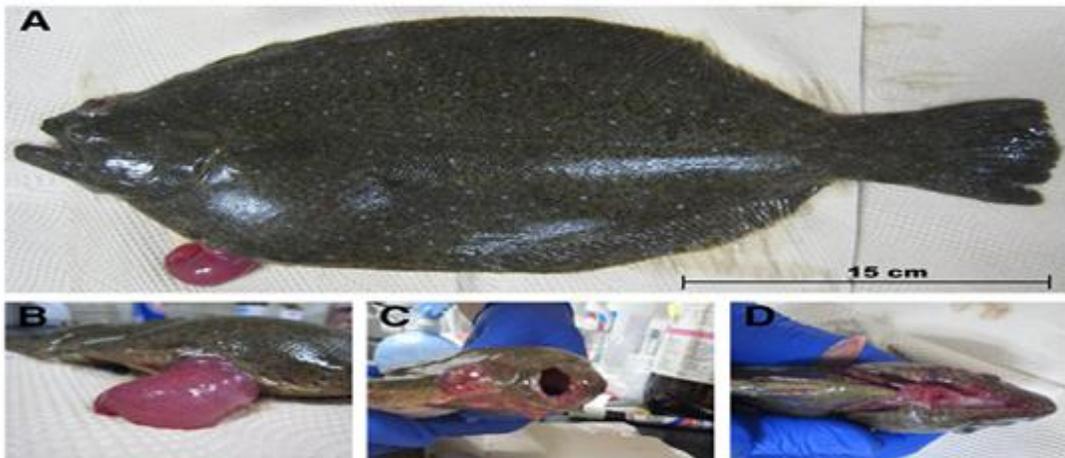


Fig. 16: External signs of olive flounder infected with *Edwardsiella tarda*. A: External lesions of diseased fish. B: Abdominal distension and rectal hernia. C: Exophthalmia and opacity of the eye. D: Peripheral hyperemia in mandible lesion (**Park et al., 2012**).

3.1.6. *Escherichia coli*

E.coli is Gram negative bacteria. Pathogenic bacteria such as *E.coli* can be transferred to oysters through handling, rising with water and inappropriate storage (Carrasco *et al.*, 2012) causing faecal contamination and at least six types of intestinal infections. Le *et al.* (2018b) reported the efficiency of bacteriophage cocktail in eliminating *E. coli* strains in the edible oyster leading to protection of consumer health. of haemorrhagic *E. coli* O157:H7 after 1 h of treatment.

3.1.7. Phage therapy of coral diseases

Coral diseases cause great harm to coral reefs throughout the world (Harvell *et al.*, 2002). Microorganisms are responsible for these diseases (Rosenberg and Ben-Haim, 2002). In a study by Efrony *et al.* (2007), lytic phages specific to bacterial pathogens that are responsible for diseases of coral were tested for phage therapy. The investigation of phage therapy of the infected coral demonstrated the success of the specific phages in controlling diseases caused by bacterial infection. The data concluded that the bacteriophages can bind to the bacterial pathogens on coral surface and they multiply with lytic activity. These phages can be kept connected with the coral and prevent the other bacterial infections. These results suggested the potential of phage therapy to control the prevalence of coral diseases (Efrony *et al.*, 2007). Photographs and electron micrographs of diseased and healthy corals, phages and bacterial pathogens used in the study are presented in Fig. 17.

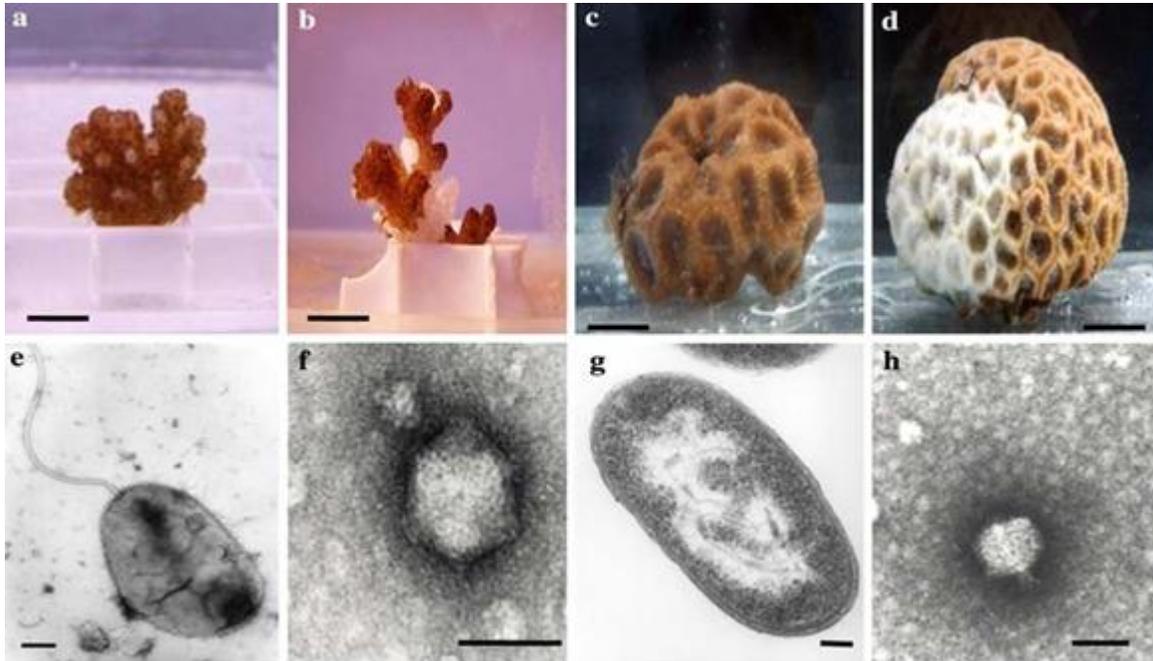


Fig. 17: Images showing corals, bacterial pathogens and bacteriophages a) Healthy, b) partially lysed *Pocillopora damicornis*, (c) healthy and d) partially lysed *Favia fava*. Negatively stained electron micrographs of *Vibrio coralliilyticus* (e), phage YB2 (f), a thin section of *Thalassomonas loyaeana* (g) and phage BA3 (h) (Efrony *et al.*, 2007).

CONCLUSION

Bacteriophages are viruses that can exist in the bacterial host. Specific phage populations differ over space and time. Moreover, bacteriophages contribute to horizontal gene transfer, evolution of bacterial species and conservation of ecological balance. Bacterial contamination in aquaculture causes great mortalities. The recent developments and future visions in the aquaculture sector is phage therapy. Administration of bacteriophage cocktails is the best appropriate application approach if large number of infected fish need to be manipulated. According to the earlier studied data, this approach introduces the regulation of severe bacterial infection in fish farming, which avoids hazard consuming of antibiotics and chemicals in aquaculture.

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