Methods and Technologies to Assess Viral Interactions in the Aquatic World

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Abstract

In this chapter, we summarize and discuss methods used to study viral interactions in aquatic environments. These methods to assess interactions between viruses, hosts and the environment (including other viruses) are operationally separated into interactions at the molecular, single-cell, and community levels. Many of these methods benefit from the methodological advancements in the field of molecular biology including the 'Omics area'. There are, however, numerous methods from other fields. We also present examples of unanswered questions along with problems solved by these questions. Finally, we conclude that viral interactions with the abiotic environment have received surprisingly little attention, so far, from a methodological perspective.

Introduction

This chapter focuses on methodological developments dealing with aquatic viral ecology, viral interactions with hosts, and virus diversity. Although the chapter discusses techniques which are particularly applicable to the study of aquatic viral ecology, many of these techniques can also be applied to viruses found in terrestrial environments (see Chapter 4). In addition, rather than being comprehensive, the chapter focusses on key concepts along with some under- or not fully considered research avenues. [217]

Techniques to study virus and virus–host interactions

In the past, studies on viruses as well as virus–host interactions in aquatic systems have suffered from technical limitations. Today, however, there is a large toolbox available for a variety of analyses in viral ecology, in particular in the probing of virus–host interactions. Elsewhere in this volume we consider insights into aquatic viral ecology (see Chapter 6) which have benefited from this toolbox, but here we summarize existing and potentially new techniques for the study of these interactions. [218]

This section is separated into three parts: (1) methods detecting organic molecules and the interactions of such molecules with viral particles (methods at the molecular level), (2) methods focusing on the localization of viruses and viral gene products or their trafficking within a cell (methods at the single-cell level) and (3) methods used at the community level, that is, as consisting of multiple species living within the

same environment. A summary of the three types of techniques including potential applications, advantages, and disadvantages is presented in Tables 15.1, 15.2, and 15.3.

Methods at the molecular level (detection of organic molecules)

Spectroscopy

Mass spectrometry (MS) measures the mass-to-charge ratio of charged molecules or molecule fragments (e.g. peptides, lipids, etc.). From this feature, it is possible to differentiate and determine a molecular mass as a means of molecule identification and quantification. MS is widely used in proteomics (Domon and Aebersold, 2006; Keller and Hettich, 2009; VerBerkmoes *et al.*, 2009; Zhou *et al.*, 2011) for peptide/protein identification, in peptide sequencing (Syka *et al.*, 2004), in relative and absolute quantification of proteins, and in the determination of post-translational protein modification. Zhou *et al.* (2011) have provided detailed information about MS-based methods. Significant progress in sensitivity and accuracy of mass spectrometers (LC MS/MS, FT-ICR MS, LTQ-Orbitrap MS, TOF MS, etc.) now enables high-throughput protein identification and quantification in the low-ppm to sub-ppm range. As well, MS has emerged as a tool used in environmental proteomics (Schneider and Riedel, 2010). Banfield *et al.* (2005) used MS for the first time to profile the proteins of a microbial community (metaproteomics) of an acid mine drainage, but no study has been performed so far for viruses.

Viruses encode multifunctional proteins that interact with host-cell proteins involved in constantly adapting to and modulating the host environment. Viswanathan and Fruh (2007) provided insights via a global monitoring on the impact of viral infection on the proteome of the host cell, highlighting interactions between viral and host proteins, in particularly using quantitative or semi-quantitative mass spectrometry-based analysis of viral and cellular proteomes. MS can also be used to identify and quantify small molecules (peptides, microRNA, RNAi, crRNA). Recently, Ankrah *et al.* (2014) employed LC MS/MS to monitor the changes of dissolved organic matter (DOM) concentration and composition of a lysate originating from the activity of a lytic phage infecting a *Sulfitobacter* strain. Their main findings were that (i) viral infection can redirect 75% of nutrients into virions (viral particles) and (ii) metabolic activity differs between virus-infected cells and uninfected cells, suggesting that viral infection alters host physiology.

Infrared (IR) spectroscopy deals with light with a longer wavelength and lower frequency than visible light. It covers a range of techniques, mostly based on absorption spectroscopy and consists of a simultaneous infrared spectroscopic measurement and an electrodeposition to collect viruses from aqueous samples. These techniques can be used to identify and study chemicals and are widely

Table 15.1 Summary of methods or approaches to study virus diversity and abundance

Methods	Applications	Advantages	Disadvantages
Methods to study virus abundance			
Direct counting			
Epifluorescence microscopy (EFM)	Enumeration of total viral abundance	Sensitive detection and accurate quantification of viruses	No reliable detection and counts of RNA and ssDNA viruses; possibility to confound small bacteria and big viruses
[225] Flow cytometry (FCM)	Enumeration of total viral* abundance and several viral subgroups	High throughput analysis, sensitive detection, and accurate quantification of viruses; better discrimination between viruses and prokaryotes compared to EFM	No reliable detection and counts of RNA and ssDNA viruses; very small viruses could fall below the detection limit
Transmission electron microscopy (TEM)	Enumeration of total viral abundance and specific morphotypes	Specific information about the morphology and the size of viral particles	Low throughput, low precision, low detection limit, time consumption and costly

Table 15.1 Continued

Methods	Applications	Advantages	Disadvantages
Indirect counting			
Plaque assays (PAs)	Enumeration of viruses specific for a selected host	Quantification of the abundance of infectious units that cause the lysis of a particular host	Host cells and viruses must be cultivable
Most-probable-number assays (MPNs)	Enumeration of viruses specific for a selected host	Quantification of the abundance of infectious units that cause the lysis of a particular host	Host cells and viruses must be cultivable
Quantitative PCR (qPCR)	Enumeration of specific viral types	Sensitive and accurate quantification of specific viral groups for which direct counting methods are limited	Knowledge of the number of copies of marker genes per genome necessary, since targeted genes can occur in several copies per genome
Fluorescence *in situ* hybridization (FISH)	Enumeration of specific viral types	Rapid and high throughput analysis	Probes are limited to specific groups; drawbacks are possible if the probe is not fully specific
Microarray	Assessment of both abundance and richness of specific viral communities	Rapid and high throughput analysis	Probes are limited to specific groups; drawbacks are possible if the probe is not fully specific
Methods to study virus diversity			
Culture-independent diversity studies			
PCR-based approaches			
Fingerprinting methods	Assessment of both abundance and richness of specific viral communities	Cost-effective, rapid	Specific for groups of viruses depending on primer design; low throughput
Cloning–sequencing	Assessment of both abundance and richness of specific viral communities	Cost-effective	Specific for groups of viruses depending on the primer design
Deep amplicon sequencing	Assessment of both abundance and richness of specific viral communities	High throughput	Specific for groups of viruses depending on the primer design
Metagenomics	Assessment of both abundance and richness estimation of total viral community	No need for prior knowledge of viruses or viral sequences present in the samples;	Possible low sequencing depth of coverage, short read length, sequencing error, microbial contamination, majority of sequences in virome has no affiliation
Pulse field gel electrophoresis (PFGE)	Assessment of genome size distribution of viruses (isolated and of communities)	No PCR steps involved	Limited to dsDNA viruses
Culture-dependent diversity studies			
Above methods in culture-independent studies	Confident and robust results on viral microdiversity of specific hosts	Circumvention of the potential problem of non-specificity of primers	Host cells and viruses must be cultivable

Table 15.2 Summary of methods used to study virus morphology, molecular structure and some virus–host interactions at molecular level

Methods	Applications	Advantages	Disadvantages
Electron microscopy			
Cryo-electron microscopy (cryo-EM)	Structural details of viral particles (e.g. topology and accessory arrangement); resolution 0.5–2 nm	No crystallisation of samples needed	Description of only the 'average' structure of entire virus population; not applicable to pleiomorphic, architecturally uniform, and virus >100 nm
Transmission electron microscopy (TEM)	Particle and cell 2D morphology imaging, e.g. used to assess virus morphology and estimate the viral burst size; also used to estimate viral infection of bacteria; resolution 0.1 nm	Classical method to visualize a virus or viral particle and provide size and morphological details	Complete sample dehydration usually required
Atomic force microscopy (AFM)	Nanoimaging 3D structure of protein and nucleic acid (i.e. secondary and tertiary structure of large RNA molecules); resolution < nm	Mechanically and electronically more straightforward than electron microscopy; inexpensive and easy to install; assessment of ultrastructure at nanoscale; analysis asymmetrical and non-uniform objects and viruses larger than 100 nm	
Crystallography			
X-ray crystallography	Protein 3D structure e.g. unveiling the crystal structure of lysin PlyC from the streptococcal bacteriophage or the structural basis for CRISPR RNA-guided DNA recognition by Cascade; resolution 2.3 nm		Crystallization of sample needed before analysis potentially affecting the state of protein/RNA structural conformation; limited to particles larger than 100 nm
Spectrometry			
Infrared spectrometry	Identification of types of virus, based on distinct vibrational fingerprints of viral components (nucleic acids, proteins, phospholipids, and other small molecules); resolution 20–30 nm	Fast and easy	Low resolution
Nuclear magnetic resonance (NMR) spectrometry	Assessment of structural details of the coat proteins and DNA of phages at the atomic resolution		Restricted to a molecular mass of up to ~100 kDa and RNAs of up to 35 kDa; gram quantities of material required; model building required for interpretation
Mass spectrometry (MS)	Used for peptide/protein identification, peptide sequencing, relative and absolute quantification of proteins, and the determination of post-translational modifications		

Table 15.3 Summary of methods or approaches to study virus–host interactions

Methods	Applications	Advantages	Disadvantages
Single-virus tracking/ imaging	Developed to follow the fate of individual viral particles, monitor dynamic interactions between virus and cellular structure/ component, study the viral entry (virus–receptor interaction, penetration, internalization), intracellular transportation, genome releasing, nuclear transport, and cell-to-cell transmission		Only applicable for virus cultures and not for environmental samples
PhageFISH	Identification of specific viruses infecting specific hosts; identification of modes of infection (lytic, lysogenic or chronic infection)	Assessment of viruses and hosts in a culture-independent way	Specific probes for each virus–host pair required
Viral-tagging	Assessment of infection of specific hosts by specific viruses	High-throughput counting of fluorescently labelled viruses	Only applicable for isolated viruses–host systems
Microfluidic digital PCR	Assessment of co-occurrence of phages and hosts	Fast screening of virus–host pairs from natural samples; independent of culturing	Possible false positive signals can occur when 1) multiple phage genes are released from a lysed cell, 2) multiple bacterial 16S rRNA genes from cells adhered to the same chamber, or 2) fluorescence signal spilled over from neighbouring chambers
Single-cell amplified genomics	Identification of individual cells and infected viruses	Viable approach to obtain genomes of unculturable viruses; assessment of viruses and hosts in a culture independent way	Low throughput, costly and time consuming

used tools for material identification and secondary structure analysis in chemistry, biology, and biochemistry. Therefore, IR spectroscopy can also be used to identify and quantify different types of viruses based on distinct vibrational fingerprints for viral components (nucleic acids, proteins, phospholipids, and other small molecules) (Vargas *et al.*, 2009). IR spectroscopy can provide information on the composition of a material within minutes. Applying IR to drinking water, which is an oligotrophic (low-nutrient) environment, low-concentrations of viral particles could be detected with good sensitivity within a few minutes. Until recently, diffraction limitations prevented nanoscale protein studies. Amenabar *et al.* (2013), however, developed a method, referred to as the Fourier transform infrared nanospectroscopy (nano-FTIR), to overcome this limitation and to map protein structure at the nanoscale with a 30-nm lateral resolution and sensitivity for individual protein complexes.

Nuclear magnetic resonance (NMR) spectroscopy exploits the magnetic properties of atomic nuclei. It determines the physical and chemical properties of atoms or the molecules in which they are contained. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the electronic structure of a molecule. NMR spectroscopy is a powerful tool to study and model the three-dimensional structure of proteins and nucleic acids at an atomic resolution (Tzakos *et al.*, 2006; Dingley *et al.*, 2008). The use of NMR as a

multiprobe method enables detailed structural studies on conformational equilibria, molecular dynamics, and the relative orientation of protein domains or proteins in a macromolecular complex as well as the identification of intermolecular interactions at protein-protein, protein-ligand, or protein–nucleic acid interfaces (Tzakos *et al.*, 2006). An example for NMR application to viruses at the atomic resolution has been the description of the structural details of the proteins and DNA of a filamentous bacteriophage (Opella *et al.*, 2008). NMR is suited to analyse binding epitopes of ligands bound to receptor proteins. It has been used to identify and characterize the binding of an antiviral compound to native human rhinovirus serotype 2 (Benie *et al.*, 2003). NMR is also suitable for RNA structural determination and thus for binding affinity interaction between microRNA and protein, or microRNA and microRNA (Scott and Hennig, 2008). Current NMR and labelling techniques for robust structure determination are now feasible for proteins with a molecular mass of up to ~100 kDa and RNAs of up to 35 kDa (Tzakos *et al.*, 2006).

X-ray crystallography

X-ray crystallography (XRC) is a tool used for identifying the atomic and molecular structure of a crystal, in which the crystalline atoms cause a beam of incident X-rays to diffract in many specific directions. By measuring the angles and intensities of these diffracted beams, a three-dimensional picture of the density of electrons within the crystal can be produced. From this electron density, the mean positions of the atoms in the crystal can be determined, as well as their chemical bonds, their disorder and various other information. XRC is a fundamental research tool that has shaped our understanding of biological structures and functions at the molecular level (Yonath, 2011). It provides the information required to create accurate three-dimensional models of organic molecules, namely methodological mapping the position of each and every atom that constitutes the studied object.

The biggest problem when using X-ray crystallography for environmental applications is that the sample needs to be crystallized prior to analysis. Crystallization may affect the native state of the analysed protein or the structural conformation of RNA. Nevertheless, X-ray crystallography remains useful in virology as indicated by the two following examples. McGowan *et al.* (2012) described the crystal structure of lysin PlyC from a streptococcal bacteriophage. They found that PlyC contain two components (PlyCA and PlyCB) which play different roles in phage-induced bacterial lysis. PlyCA contains a glycoside hydrolase domain in addition to the previously recognized cysteine, histidine-dependent amidohydrolases/peptidases (CHAP) catalytic domain. The presence of eight cell wall-binding domains together with two catalytic domains may explain the extraordinary potential of the PlyC holoenzyme to target bacteria. X-ray crystallography has also revealed the composition and structure of what is known as Cascade, consisting of CRISPR-associated Cas proteins and crRNA, and the structural basis for CRISPR RNA-guided DNA recognition by Cascade (Jore *et al.*, 2011).

Electron microscopy

An electron microscope uses a beam of accelerated electrons as a source of illumination. Since the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, the electron microscope has a higher resolving power than a light microscope and can reveal the structure of objects or features of objects which are smaller than a wavelength of light. Electron microscopy (EM) is a classical method used for the visualization of viruses and virus-like particles and provides morphological details (Ackermann, 2012). There are many other applications of EM at the molecular level. For example, immuno-EM allows the detection of specific proteins in ultrathin tissue sections. Antibodies labelled with heavy metal particles (e.g. gold) can be directly visualized using transmission electron microscopy (TEM). Viruses and viral proteins can be localized within cells using such techniques.

Transmission electron microscopy (TEM) is the traditional method used to describe morphological features of isolated viruses. It offers also the possibility to describe viruses on organic particles as long as these particles are not electron dense. Upon specific collection of organic particles and embedding them into a resin, ultrathin sections can be made. Subsequently the viruses can be enumerated (Peduzzi and Weinbauer, 1993). Also, the fraction of host cells containing mature virions can be quantified (Proctor and Fuhrman, 1991). Using

conversion factors and models, an estimation of the percentage of infected cells and viruses mediated mortality can be made. Scanning electron microscopy (SEM) in principle is also applicable to the study of viral particle interactions. So far, however, only specific and potentially large viruses can be detected by SEM.

In Cryo-EM, also known as electron cryotomography, multiple tilted 2D EM images made at cryogenic temperatures are combined to obtain a 3D reconstruction of a sample. It is based on automated collection, cataloguing and the elaboration of mathematical techniques in order to analyse images of viral particles. These are then used for the reconstruction of viral particle structures from a collection of particle observations, taken from multiple orientations/angles. Cryo-EM can thus be used to obtain structural details of viral particles (e.g. topology and accessory arrangement) at subnanometre scale. This method has been used for example in the characterization the marine siphovirus Vibrio phage SIO-2 (Lander *et al.*, 2012).

Atomic force microscopy

Atomic force microscopy (AFM) is a very high-resolution type of scanning probe microscopy that forms images of surfaces using a mechanical probe on a cantilever. When the tip of the probe is brought into the proximity of a sample surface, the forces between the tip and the sample lead to a deflection of the cantilever, which can be measured. Resolution on the scale of fractions of a nanometre has been obtained. This technique has been reviewed by Kuznetsov *et al.* (2011) for the application of imaging viruses and virus-infected cells. Atomic force microscopy is also a promising technique for the nano-imaging and the determination of the overall 3D structure of large RNA molecules. Its recent application on a hepatitis C virus (Gilmore *et al.*, 2014) opened up a whole new avenue for the study of the secondary and tertiary structure of long RNA molecules. Like cryo-EM, AFM does not require the crystallization of viral particles and has a similar level of resolution. It can be used to analyse viruses that do not have symmetrical or uniform architectures, while this is not the case for cryo-EM and X-ray crystallography (which are both based on an average estimation of symmetrical viral structures). Moreover, AFM can also be used for the analysis of giant viruses with sizes larger than 1000 nm, whereas both cryo-EM and X-ray chromatography are limited to smaller particles (Kuznetsov *et al.*, 2011).

Summary and outlook

Only NMR and AFM allow for structural investigations of nucleic acids, as they provide high resolution. It is noteworthy that only MS and EM have been employed for environmental samples so far; other techniques require large amounts of material for robust analysis or detection, and have therefore been only used in laboratory studies with cultured viruses. An improvement of these techniques is required for environmental applications within complex communities, to allow *in-situ* and reproductive analysis from as little as, for example, 1 ml of raw water.

Methods at the single-cell level

Electron microscopy

Transmission EM (TEM) reveals 2D images and requires staining with heavy metal dyes or shadowing for contrast enhancement. By examining and quantifying viruses in cells, TEM is also used for the estimation of virus-mediated bacterial mortality (Proctor and Fuhrman, 1990; Weinbauer *et al.*, 1993, Binder, 1999). Modification and improvement of TEM resolution have been developed to meet different needs of application, such as seen with what is described as high-resolution TEM (HRTEM).

Fluorescence *in situ* hybridization-based approaches

Fluorescence *in situ* hybridization (FISH) is a powerful tool for the single cell detection of different bacterial types and has also been applied to viruses. Allers *et al.* (2013) developed the phageFISH method, consisting of an improved version of the geneFISH protocol (Moraru *et al.*, 2010) that increases gene detection efficiency from 40% to >92% at the single-cell level. This method was optimized for the detection and visualization of intracellular as well as extracellular phage DNA and allows a simultaneous identification and quantification of host cells during all stages of infection. Briefly, it uses a catalysed reporter deposition (CARD)-FISH protocol (Pernthaler *et al.*, 2002; Amann and Fuchs, 2008) to link, on the

one hand, the florescence-labelled rRNA detection and, on the other hand, the detection of viral DNA. Visualization is carried out using epifluorescence microscopy. This approach offers the opportunity to link viruses and hosts in a culture-independent manner and to study the virus–host interaction at the single-cell level. It is based on an appropriate probe design, which is achieved given *a prior* knowledge of phage and host genes. This method has been validated using a marine virus–host model (e.g. *Pseudoaltermonas* sp. strain H100 and its phage PSA-HP1, Allers *et al.*, 2013), but has not yet been applied to field samples. It could be potentially useful for environmental surveys and lineage-specific population ecology of free phages (such as for well documented viruses, like cyanomyoviruses, T4-like myophages, etc.).

Kenzaka *et al.* (2010) developed the 'cycling primed *in situ* amplification fluorescence *in situ* hybridization' (CPRINS-FISH) method for studying virus-mediated gene transfer in freshwater ecosystems. In this method, viruses were labelled via the *gfp* or *bla* gene cloned into the genome and detected by using epifluorescence microscopy. Direct viable counting combined with CPRINS-FISH revealed that more than 20% of the cells carrying the transferred gene retained their viability. This brought into question the previous assumptions that a proportion of transferred genes inside recipient cells may be destroyed. These results, however, suggested that DNA exchange among bacteria via phages in natural aquatic environments may be more frequent than previously thought.

Single virus tracking

Single-virus tracking (Brandenburg and Zhuang, 2007) is a FISH method in living cells and offers the possibility to detect individual viruses in living cells. Using this method, both viral and cellular components (e.g. protein, genome, or membrane, etc.) are labelled using specific fluorescent probes. The labelled viruses then can be visualized in live cells by using the fluorescence microscopy (e.g. epifluorescence microscopy, confocal laser scanning microscopy, or total internal reflection fluorescence microscopy). This single-virus imaging method allows following of the fate of individual particles, monitoring dynamic interactions between viruses and cellular structures/components, and studying viral entry (virus–receptor interaction, penetration, internalization), intracellular transportation, genome release, nuclear transport, and cell-to-cell transmission (Marsh and Helenius, 2006; Brandenburg and Zhuang, 2007; Sun *et al.*, 2013). For example, Hübner *et al.* (2009) monitored the movement of Gag-GFP in HIV-infected cells and found that the Gag proteins migrate and assemble into button-like structures adjacent to neighbouring cells. The authors further showed that the button-like Gag structures could enable cell-to-cell transmission and infection of HIV.

Flow cytometry-based approach

In addition to phageFISH, Deng *et al.* (2012) also developed a viral-tagging (VT) method that uses flow cytometry (FCM) to count fluorescently labelled viruses. This FCM-based approach allows high-throughput detection and sorting of co-occurring viruses and hosts. This method was tested and validated using two cultivated hosts (the cyanobacterium *Synechococcus* and the gammaproteobacterium *Pseudoalteromonas*) and their phages (podo-, myo-, and siphoviruses) by comparing results with a conventional method (liquid plaque assay) (Deng *et al.*, 2012).

Microfluidic digital PCR

The microfluidic digital polymerase chain reaction (PCR) technique (Tadmor *et al.*, 2011) serves to link single bacterial cells harvested from a natural environment to a viral marker gene, i.e. to detect an infected cell. Briefly, both a viral gene and the bacterial 16S rRNA gene are amplified using specific primers in each microfluidic PCR chamber that ideally contains only a single bacterial cell. They can be thereafter labelled using two fluorescence-labelled probes, one for the virus and another for the host. The co-localization of virus and host can thus be visualized and virus–host pairs can be identified by sequencing (Tadmor *et al.*, 2011).

Single-cell/virus-genomic approach

Using single-cell genomics, viral DNA can also be sequenced (Ishoey *et al.*, 2008; Woyke *et al.*, 2010; Stepanauskas, 2012), thus providing a means to reveal organismal interactions in uncultivated microbes. Yoon *et al.* (2011) used fluorescence-activated cell-sorting to obtain individual heterotrophic protist cells from a 50-ml seawater sample, and then applied whole-genome-sequencing of three

sorted and single uncultivated marine protist cells (Picobiliphyta). They showed that genome data from one cell were dominated by sequences from a widespread single-stranded DNA virus, MS584–5, which was closely related to *Nanoviridae*, using the Rep gene as the phylogenetic proxy. Results showed a lytic interaction with the host. This virus was absent from the other two cells, however. Both cells also contained non-eukaryote DNA derived from marine Bacteroidetes and large DNA viruses. This single-cell-genomic approach (i) revealed potentially complex biotic interactions among previously uncharacterized marine microorganisms, and (ii) provides a new approach to study, without cultivation artefacts, the viral interactions with their hosts (protists and prokaryotes) *in situ*.

Summary and outlook

PhageFISH allows *in situ* tracking of viruses in the environment. Viral-tagging (VT) and microfluidic PCR do not allow *in situ* analysis but can instead be used to track viruses at different time points in virus–host systems. Additionally, the VT method enables high-throughput analysis as it employs sorting by FCM. Using VT, PhageFISH or microfluidic PCR requires targeted virus and host sequences to be known for probe or primer design. In contrast, the single-cell/virus genomics approach is not limited by this constraint.

Interactions at the community level

Quantifying the virosphere

The focuses of viral ecology have evolved during the past decade. After a bloom of viral abundance studies lasting about two decades, the interests of viral ecology have shifted from the quantitative to the qualitative exploration of the viriosphere. Consequently, there has been little development of the quantification techniques. Within the reports of viral and host abundance, inference of interactions was made using the virus-to-prokaryote ratio (VPR), using the numerical dominance (or lack thereof) of viral particles over hosts as indicative of high/low viral dynamics. This has since been shown to be over simplistic (Parikka *et al.*, 2016). Before the use of direct counting methods, viral abundance in aquatic ecosystems was investigated using indirect methods by plaque-essay on agar plates (yielding plaque-forming units (PFU)) or by the most-probable-number assays (MPN) in liquid medium. The discovery of the high incidence of viral particles in various marine waters by Bergh *et al.* (1989) launched numerous investigations of viral abundance in a variety of aquatic ecosystems using different direct counting techniques. The methods commonly used are TEM, epifluorescence microscopy (EFM) and FCM, which were developed for viral enumeration roughly in that order. Since it is not always possible to prove a viral origin by using these methods, one finds also the term virus-like particle (VLP) in the literature.

While the majority of current studies presenting data on viral numbers used EFM and FCM, due to methodological problems with the TEM approach a new technique also using TEM has been recently developed. Quantitative TEM (qTEM) (Brum *et al.*, 2013) is based on the use of an air-driven ultracentrifugation of viral samples onto hydrophilic-rendered Formvar copper grids (Brum and Steward, 2010). The method, described by Hammond *et al.* (1981) for animal viruses and as subsequently adapted to phage enumeration (Maranger *et al.*, 1994), has been redescribed for prokaryotic virus quantification and evaluated by Brum and co-workers (Brum and Steward, 2010; Brum *et al.*, 2013). The authors report no significant differences between the morphological data of viruses between absorption of marine samples onto grids and the deposition of viruses directly onto grids by qTEM hence, no biases are expected. Viral enumeration using qTEM thus allows morphological data analysis in addition to providing quantitative data on viruses, which represents an advantage of this direct counting method compared to EFM and FCM.

Viral enumeration using EFM is essentially a variation of the methodology applied originally to prokaryotic enumeration, where cells are captured on filters, stained with fluorescent dyes, and counted using a microscope (Francisco *et al.*, 1973; Daley and Hobbie, 1975). As this can be laborious, Cunningham *et al.* (2015) developed the 'wet mount' method, where stained samples are mounted directly onto slides without first capturing viral particles onto a filter. In this method, the sample is first concentrated (if necessary), stained with a fluorescent dye and then a known concentration of silica microsphere beads is added prior to observation. The sample is then wet mounted to

a slide and the viral numbers are counted directly. Their concentration in the sample is finally inferred from their ratio in relation to added microsphere beads. This method is rapid, accurate and ca. 500-fold less expensive in material costs compared to the classical filter-mounting method.

Requiring more equipment and expertise than EFM, flow cytometry is also based on the staining of the nucleic acids of viral particles using fluorescent dyes. FCM has the distinct advantage of a rapid and high-throughput analysis of samples over TEM and EFM, processing at a rate of thousands of events per second (Brussaard *et al.*, 2010; Wang *et al.*, 2010) and the possibility to distinguish different viral populations (Marie *et al.*, 1999; Brussaard *et al.*, 2000). As virtually all types of samples can be eluted into water after sample treatment, EFM and FCM are applicable to all types of samples. For viral enumeration in aggregates (e.g. aquatic snow), another technique was proposed by Luef *et al.* (2009) and validated by Peduzzi *et al.* (2013) as an alternative to the three mentioned counting methods: confocal laser scanning microscopy (CLSM). Preceding enumeration, the matrix material of aggregates is stained using lectin and prokaryotic cells and viral particles are dyed using SYBR Green I. Cryosections are also performed on larger aggregates, allowing better detection and distribution of viruses, bacteria and aggregate constituents. Although enumeration seems to be somewhat difficult due to problems with digital image analysis of the CLSM pictures, confocal laser microscopy offers the benefit of the visualization of viral particle and prokaryotic cellular distribution within aggregates, and thus is a promising avenue for the ecological studies of aquatic aggregates. Allen and colleagues (2011) combined genomics with CLSM to sort and sequence single viral particles, yielding 'Single Virus Genomics'. Using a mixed viral assemblage of fluorescently dyed *Escherichia coli* phages λ and T4, they sorted viral particles, using a forward scatter photomultiplier tube for more sensitivity, into agarose beads applied to 'multi-well' microscope slides. The nanolitre droplets containing the sorted virions were overlaid with additional agarose for embedding and stabilizing the particles for CLSM. The individual virions were then visualized with CLSM and beads, with only a single virion chosen for further analysis. Whole genome amplification via the multiple displacement amplification (MDA) was performed *in situ* and subsequently sequenced.

Most of the developments of methods related to EFM and FCM has been in sample preparation in order to better distinguish viral particles from background noise. Little has been done, however, to enhance the detectability of viruses with genomes other than double-stranded DNA. Regardless of the stainability of single- and double-stranded DNA and RNA (Tuma *et al.*, 1999; Shibata *et al.*, 2006), ssDNA and RNA viruses (and viruses of small genomes in general) are either scarcely detected or undetectable (Brussaard *et al.*, 2000; Holmfeldt *et al.*, 2012). Although it has been presumed that dsDNA phages are the prevailing viruses in aquatic ecosystems (Steward *et al.*, 1992; Breitbart *et al.*, 2002; Weinbauer, 2004; Comeau *et al.*, 2010), more recent studies suggest an unexpected importance of ssDNA and RNA viruses (Angly *et al.*, 2006; Lang *et al.*, 2009; Rosarioa and Breitbart, 2011; Roux *et al.*, 2012). Therefore, the future of the improvement in FCM probably lies in the development of methods to detect ssDNA and RNA viruses.

In medicine, developments for the detection of RNA viruses, such as HIV, have been made by adding markers, such as combinations of beads and antibodies. In this technique either several (Kim *et al.*, 2009) or a single (Arakelyan *et al.*, 2013) virion is attach to magnetic beads and then revealed by specific antiviral fluorescent antibodies, giving the name 'Flow Virometry'. Gaudin and Barteneva (2015) developed the 'Flow Virometry Assay' in which they detected and sorted ssRNA Junin-viruses (a causative agent of Argentine haemorrhagic fever), while being able to retain virion infectivity in the process. The authors used a flow cytometer equipped with a powerful laser (300 mW and 488 nm), a digital focusing system (DFS), and using the forward scatter channel option for the multiplier tube (FSC-PMT). By optimizing the parameters of the DFS, a picomotor-driven focusing device that adjusts the beam in order to obtain a smaller focal spot, they were able to detect small lipid microvesicles. The application of this technology to viral ecology could enable the exploration of the ssDNA, dsRNA and ssRNA viruses, as well as other elements of horizontal gene transfer, such as gene transfer agents (GTA), membrane vesicles (MV) and VLP. As pointed out by Forterre *et al.* (2013), the presence of the aforementioned

elements of horizontal gene transfer elements are likely to interfere with the counts of *bona fide* viral particles in a given sample. There is currently few data on the abundance of MVs, GTAs, BLPs and free nucleic acids, although their ubiquitous production in aquatic ecosystems has been established (Mashburn-Warren and Whiteley, 2006; McDaniel *et al.*, 2010; Chiura *et al.*, 2011; Gaudin *et al.*, 2013; Lang *et al.*, 2012).

Fingerprints

DNA fingerprinting describes a collection of techniques originally developed to identify individual organisms but later adapted for species identification and studies of diversity. Originally accomplished with restriction analysis and Southern blotting techniques, PCR has become a core technique. DNA sequencing technologies especially those used in metagenomics analysis are also used.

Although several biases can be associated to the use of fingerprint methods, these techniques remain very useful, as they are cost-effective and reproducible (e.g. denaturing gradient gel electrophoresis DGGE; Zhong and Jacquet, 2013; Zhong *et al.*, 2014). In addition to fingerprinting methods, sequencing the amplicons (using next generation sequencing (NGS), i.e. deep-sequencing or ultra-deep sequencing) of the cloned amplicons (using traditional Sanger sequencing method, i.e. cloning–sequencing) have been widely used. The classical cloning–sequencing method is based on the construction of a clone library for amplicon DNA fragments with a plasmid vector for its expression in a host cell (e.g. *E. coli*, etc.) and clones are then sequenced by using the Sanger method (Zhong *et al.*, 2015). With the improvements in sequencing technologies in recent years, the high throughput 'next generation sequencers' (e.g. 454 pyrosequencing, Illumina, SOLiD, Helixo, PacificBio), it is now possible sequence millions/billions of DNA fragments per day, with costs that are increasingly affordable. Such an approach has the advantage of revealing what is rare, i.e. the 'rare biosphere'(Sogin, 2006), as it allows deep sequencing (Simon and Daniel, 2011).

Randomly amplified polymorphic DNA (RAPD)-PCR and separation by gel electrophoresis of viral concentrates from isolates or natural communities yield a simple fingerprint based on size patterns (Winget and Wommack, 2008). These fragments can be used as probes or sequenced. The same can be done for the host. A high seasonal variability of deep-sea viruses as well as the co-variation with host community structure has been observed using such a technique (Winter and Weinbauer, 2010).

Pulsed field gel electrophoresis (PFGE) allows fingerprinting virioplankton based on size fractionation of intact genomic DNA. This technique has proved on many occasions, since the pioneer study of Wommack *et al.* (1999), to be very powerful in giving a first insight on genome size distribution in a variety of aquatic environments. It provided the first data on viral community dynamics related to their genome sizes in an aquatic environment. PFGE, however, can only provide a rough minimum estimate of the virioplankton diversity (Parada *et al.*, 2008). Indeed, it can only be used for dsDNA viruses. Also, PFGE can only reveal abundant groups and hence is limited for the detection of minor groups (Filippini and Middelboe, 2007, Colombet *et al.*, 2006, Zhong *et al.*, 2014). Because a good PFGE performance requires a large amount of DNA (corresponding to ca. 10^9 viruses per plug), insufficient DNA loads may make bands and thus viruses invisible. To obtain a sufficient concentration of VLPs, additional steps are required to concentrate viruses, which can also produce biases. Moreover, one PFGE band, representing a subpopulation of dsDNA viruses (characterized by having the same genome size), may contain several genetically and morphologically different viruses or viral groups, and the composition of this subpopulation (band) may vary with time (Jamindar *et al.*, 2012). It is noteworthy that PFGE can be very powerful if combined with PCR when investigating the diversity of specific viral groups, based on the analysis of the DNA bands isolated from PFGE gel slices. This type of analysis has been useful in detecting connections between virus phylogenetic affiliation and genome size (Sandaa and Larsen, 2006; Sandaa *et al.*, 2008; Jamindar *et al.*, 2013; Zhong *et al.*, 2014).

PCR is a sensitive tool that has been successfully used to explore viral diversity using degenerate primers targeting phylogenetic markers. Unlike prokaryotes and eukaryotes, for which universal genes and primers exist, the application of PCR to viruses is trickier. Although no common genes can be found for all viruses, some conserved core genes

exist in different viral groups that can be used as genetic markers for these viral groups (Rohwer and Edwards, 2002). The differences in sequences found for these genes correspond to potentially different genotypes, and the number of genotypes can be used as a proxy of viral species richness (Weinbauer and Rassoulzadegan, 2004). These group-specific [219] gene markers are, for instance, *g20*, *g23*, and *g43* for T4-like *Myoviridae* including cyanophages (Fuller *et al.*, 1998; Zhong *et al.*, 2002; Desplats *et al.*, 2003; Filée *et al.*, 2005; Comeau *et al.*, 2008; Sullivan *et al.*, 2008; Marston *et al.*, 2013); *polA* for T7-like *Podoviridae* (Breitbart *et al.*, 2004; Labonté *et al.*, 2009; Chen *et al.*, 2009; Dekel-Bird *et al.*, 2013); *psbA* for most isolated cyanophages (Suvillan *et al.*, 2006); *polB* and *mcp* for nucleocytoplasmic large DNA viruses (NCLDV) (Chen *et al.*, 1996; Larsen *et al.*, 2008). To analyse PCR amplicons and assess viral diversity, fingerprinting methods (see above), cloning–sequencing (clone library construction followed by Sanger sequencing), or deep-sequencing (amplicons tagged plus massive sequencing by NGS can be used (for instance Zhong *et al.*, 2013, 2014, 2015).

Metagenomics, metatranscriptomics, metaproteomics, metabolomics

Metagenomics is the study of genetic material recovered directly from environmental samples. The broad field may also be referred to as environmental genomics, ecogenomics, or community genomics. Likewise, studies at the community levels are called metatranscriptomics when gene expression is studied based on RNA sequence and metaproteomics when gene expression is studied for the proteins. Metatranscriptomics and metaproteomics studies have not been yet performed at the community levels. Metabolomics is the scientific study of chemical processes involving metabolites. More specifically, metabolomics is the study of the unique chemical fingerprints that specific cellular processes leave behind. The metabolome represents the collection of all metabolites, which are the end products of cellular processes. Gene expression data based on mRNA along with proteomic analyses reveal the set of gene products being produced within a cell at a given moment or under a given set of environmental conditions, data that represents one aspect of cellular functions. Conversely, metabolomic profiling can give an instantaneous snapshot of the physiology of the cell. Metabolomics at the community level (community metabolomics or 'metametabolomics') remains unapplied to viruses.

Metagenomics is a powerful technology, developed during the last two decades, which allows both phylogenetic and/or functional studies on microbial/viral communities using total DNA/RNA, extracted from the environment (see Chapter 5). One of the approaches is the whole metagenome sequencing using the Sanger method to sequence the shot-gun libraries with the goal of studying microbial/viral phylogenic and functional diversity. Using this approach, it was shown that marine viruses are extremely diverse and more than half of the sequences obtained were unknown (Breitbart *et al.*, 2002; 2004; Bench *et al.*, 2007; Williamson *et al.*, 2008). These studies thus indicated the great force and potential of sequencing for examining microbial community diversity. Recent high-throughput sequencing technologies have provided a rapid and robust resolution for exploring the vast taxonomic diversity of viruses as they enable the direct sequencing of DNA without clone library construction and associated biases. The first application of metagenomics for the study of aquatic viruses was carried out on seawater-samples by Angly *et al.* (2006). Compared to other studies (Breitbart *et al.*, 2002, 2004; Bench *et al.*, 2007), which used the traditional sequencing method, this approach lead to the discovery of a large and unknown viral diversity. Since then, it has been applied for studying viral communities in oceans (Angly *et al.*, 2006), lakes (López-Bueno *et al.*, 2009; Lauro *et al.*, 2011; Roux *et al.*, 2012; Brum *et al.*, 2015a,b; Skvortsov *et al.*, 2016; Aguirre de Cárcer *et al.*, 2016), reclaimed water (Rosario *et al.*, 2009) and desert ponds (Fancello *et al.*, 2013), as well as for revealing viral–host interactions (Reyes *et al.*, 2010; Rodriguez-Brito *et al.*, 2010).

Unlike PCR-based methods, the metagenomic approach does not rely on prior knowledge of viruses or viral sequences present in the samples (Mokili *et al.*, 2012). Such an approach is therefore well adapted for the investigation of the diversity of viruses, for which no universal phylogenetic marker exists. The total available virome sequences make the study of viral taxonomic diversity easier and more comprehensive (Rosario and Breitbart, 2011). Hence, viral metagenomes allow the examination of the whole virioplankton diversity,

providing both (i) functional and phylogenetic diversity analysis as well as (ii) abundance and richness at the same time. Although the application of NGS in metagenomic has extended our ability to assess multiple metagenomes and increased our knowledge on environmental microbial diversity, it currently also suffers from problems such as those associated to the low sequencing depth of coverage, short read length, sequencing error, microbial contamination, and from the fact that the majority of sequences in virome typically are found to have no known affiliation (Mokili *et al.*, 2012). Despite all drawbacks mentioned above, the current high-throughput sequencers are powerful and especially so as the stability, fidelity, high-throughput characteristics, read-lengths, and single-molecule sequencing technologies improve.

It is also noteworthy that methods exist to assess the diversity of RNA viruses (Culley *et al.*, 2006), by which the RNA virome is firstly reverse-transcribed to complimentary DNA prior to sequencing. Recent studies have revealed that, in some situations, virioplankton could be largely represented by RNA viruses (Culley *et al.*, 2006; Lang *et al.*, 2009; Steward *et al.*, 2013). Steward *et al.* (2013) investigated both RNA and DNA viromes of the California coastal seawater and demonstrated that RNA viruses are as numerous as DNA viruses, accounting for 38–60% of total viral abundance. Molecular surveys using degenerate primers to target the RNA-dependent RNA polymerase gene of picorna-like viruses have shown that, in addition to the handful of isolates, a very diverse pool of uncultivated picornaviruses exist in seawater (Culley *et al.*, 2003; Culley and Steward, 2007; Gustavsen *et al.*, 2014). The quantification of these RNA viruses, however, has remained unachieved so far.

While viral metagenomics have been applied more and more during the last decade, only a few studies have been made available using transcriptomics (Brum and Sullivan, 2015). To give a recent example, Lin *et al.* (2016) studied the transcriptomic responses of *Prochlorococcus* infected by a cyanomyovirus under phosphorus limitation, a strong selective force in the ocean. They could reveal that transcripts of the phosphorus acquisition genes such as *pstS* in the uninfected cells were enriched after phosphorus limitation but also in the infected cells. By contrast, other genes, such as ATP synthetase and ribosomal protein genes, were depleted in uninfected cells after phosphorus limitation but were enriched in infected cells. Their study also revealed that phage *pstS* transcript number per cell was almost 20 times higher than the host copy, suggesting this may help to maintain the host phosphate uptake rate during viral infection.

Summary and outlook

Fingerprints allow for rapid detection of changes in the viral groups making up communities found in experimental set-ups and systems, and along environmental gradients such as temperature, light, water depth (pressure), salinity, pCO2 levels or trophic status of the system. Some fingerprints can also provide sequence information (e.g. from excised DGGE or RAPD-PCR bands) or can be combined with subsequent approaches to obtain sequence information (e.g. excised PFGE bands + fingerprinting + sequencing). There are no common genes for viruses, not even for the monophyletic group of the *Caudovirales* (Ackermann 1998). Hence, unless genomic DNA (or RNA) is the target, fingerprints are only targeted against specific viral groups, and this limits the information obtainable for entire communities. Also, the majority of environmental sequences, such as those obtained by metagenomic approaches, have no homologues in databases of isolated viruses. Hence, we do not know what most of the sequences encode.

Conclusions

Overall, the number of methods used to analyse virus interactions at the molecular, cellular and community level have increased tremendously during the last decades. Recently, new analyses to deal with large-scale data sets have allowed assessment of the potential for co-occurrence of organisms and non-living environmental conditions including the role of viruses in the ocean. For example, the implication of viruses associated with carbon export in nutrient-poor regions of the ocean has been described (Chow *et al.*, 2013).

The toolbox for the more direct study of the interactions between viruses and the abiotic environment nevertheless is poorly filled, especially in non-aquatic systems. Epifluorescence microscopy has been used extensively to quantify viruses in

the water column and in the sediments of aquatic systems and represents a golden standard, of sorts, against which other methods such as flow cytometry (Brussaard *et al.*, 2010) have been tested. Epifluorescence microscopic enumeration has also been used to roughly quantify the proportion of 'free-living' viruses and the proportion of viruses attached to particles such as debris (marine and lake snow) or suspended sediment material (Weinbauer *et al.*, 2009). Upon selective collection of organic particles and separation of viruses from the organic matrix, viruses can be counted more specifically. Recently, epifluorescence microscopy based approaches to assess viral production and virus-mediated mortality of prokaryotes (virus reduction approach) have been applied to organic particles (Bettarel *et al.*, 2016). Further improvements will be required for the detection of ssDNA, dsRNA and ssRNA viruses as well as elements of horizontal gene transfer. For these, some methodologies exist in the medical field.

Laser scanning microscopy (LSM) has been used to detect viruses on and within particles such as marine, lake and river snow, soot or desert dust and obtain digitally created high-quality images. This method is useful to estimate viral abundance on particulate material, such as transparent exopolymeric particles (TEP) and other types of aquatic aggregates (e.g. Weinbauer *et al.*, 2009; Peduzzi *et al.*, 2013), however it also allows assessment of the distribution of viruses on particles. In combination with specific markers, the distribution of specific viruses or viral groups on and within particles thus could be assessed in the future. An LSM picture of a river aggregates in combination with lectin detection of the aggregate matrix, with viruses, is shown in Fig. 15.1. It can be anticipated that such methods, and other techniques such as AFM, will increase our knowledge how viruses interact e.g. with organic and inorganic particles.

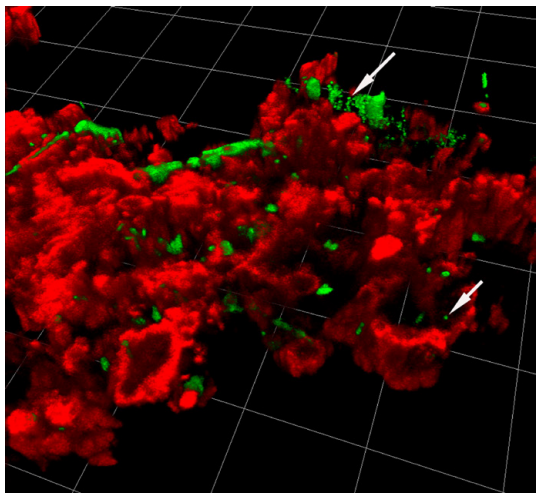

Figure 15.1 3D volume reconstruction of a river aggregate by confocal laser scanning microscopy. Arrows point to viruses. Green: Nucleic acid (staining by SYBRGreen I); red: glycoconjugates (detection by lectin).

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