Fingerprinting aquatic virus communities

Ruth-Anne Sandaa,[1]* Steven M. Short,[2] and Declan C. Schroeder[3]
[1]University of Bergen, Department of Biology, Bergen, Norway
[2]University of Toronto Mississauga, Department of Biology, 3359 Mississauga Road North, Mississauga, Ontario, Canada
[3]Marine Biological Association of the UK, Citadel Hill, Plymouth, UK

Abstract

To circumvent the limitations of cultivation-based studies of complex microbial communities, molecular fingerprinting techniques such as pulsed field gel electrophoresis (PFGE) and denaturing gradient gel electrophoresis (DGGE) have been used to examine their richness, diversity, and dynamics. PFGE is based on the electrophoretic separation of extremely large DNA, raising the upper size limit from 50 kb (standard agarose separation) to well over 10 Mb. This technique has been used to separate aquatic virus genomes ranging in size from tens to hundreds of kilo base pairs (kb); aquatic virus genomes range from 15 to 630 kb, with the majority between 20 and 80 kb. DGGE, on the other hand, is based on the electrophoretic separation of PCR-amplified gene fragments of similar sizes, but differing in base composition or sequence. In this chapter, we provide a brief overview of each of these methods and their application to the study of aquatic viruses. We describe some of the common equipment, reagents, and procedures involved, and conclude by briefly considering some of the strengths and weaknesses of each method.

Introduction

To circumvent the limitations of cultivation-based studies of complex microbial communities, gene-based molecular techniques were developed, and the discipline *molecular microbial ecology* was born. The earliest studies in this new discipline created a wealth of information about the species richness of microbial communities. Because these studies were based on relatively labor-intensive cloning and sequencing approaches, however, they were often limited to investigations of only a few select samples. The application of genetic fingerprinting techniques such as pulsed field gel electrophoresis (PFGE) and denaturing gradient gel electrophoresis (DGGE) overcame these limitations by permitting community composition comparisons of multiple samples. DGGE, a PCR-based separation technique,

gave microbiologists the tools to examine genetic diversity of previously uncharacterized mixed microbial populations (Muyzer et al. 1993). PFGE, initially developed to separate yeast chromosomes (Schwartz and Cantor 1984), was successfully exploited to characterize microbial populations based on their genome sizes. Over the last decade or so, aquatic virologists have applied these two fingerprinting techniques to great effect.

PFGE studies explored the community dynamics of dsDNA viruses in natural aquatic environments (Jiang et al. 2004, Riemann and Middelboe 2002, Sandaa and Larsen 2006, Sandaa et al. 2003, Steward et al. 2000, Wommack et al. 1999). The method was first used to characterize bacteriophages in sheep rumen, and it was demonstrated that one PFGE band consisted of DNA from a single phage genotype (Swain et al. 1996). In the marine environment, DNA from virus-like particles were found to range in size from 15 to 630 kb, with most of the DNA present in the range of 20–80 kb (Sandaa 2008, Steward et al. 2000). Use of PFGE to study marine dsDNA viral diversity has shown substantial temporal and spatial changes in genome sizes, and the results emphasize that the highly dynamic viral communities are tightly linked to the dynamics of the host populations (Larsen et al. 2001, 2008; Øvreås et al. 2003; Steward et al. 2000; Wommack et al. 1999). In mesocosm studies, PFGE was used together with analytic flow cytometry to demonstrate a distinct dynamic link between two specific large dsDNA viruses, EhV and CeV, and their respective hosts *Emiliania huxleyi* and *Crysochromulina ericina* (Castberg et al. 2001; Larsen et al. 2001, 2008). Further, this

method was used in conjunction with DGGE to investigate the relationship between bacteriophages and their hosts in nutrient-manipulated mesocosms and the effect of both top-down and bottom-up control of the bacterial community (Øvreås et al. 2003).

Among the first reports of applying DGGE as a tool for aquatic virus research were studies of marine cyanophage diversity. Wilson et al. (2000) initiated this work by examining the diversity of a viral capsid protein (g20) in marine environments. Later, Frederickson et al. (2003) adopted this DGGE-based technique and demonstrated that patterns in cyanophage communities were related to the physical structure of the water column in marine environments. Other studies that were also based on this DGGE approach revealed that cyanophage diversity was temporally variable in Lake Bourget, France (Dorigo et al. 2004), and that highly similar phage sequences could be obtained from a variety of marine and freshwater samples (Short and Suttle 2005). More recently, DGGE was used to study the diversity of g20 in Norwegian coastal waters, revealing surprising genetic diversity and seasonal shifts in cyanophage communities (Sandaa and Larsen 2006). Around the same time that the first reports of DGGE analysis of cyanophage genes appeared in the literature, other investigators were using DGGE to examine the diversity of DNA polymerase genes belonging to viruses that infect eukaryotic algae, i.e., the phycodnaviruses (Short and Suttle 1999). Eventually, these researchers used DGGE-based methods to show that some phycodnaviruses were widely distributed in nature (Short and Suttle 2002), and some persisted in British Columbia coastal waters throughout most of a year-long study (Short and Suttle 2003). Similarly, to study the population dynamics of viruses that infect the bloom-forming alga *E. huxleyi*, Schroeder et al. (2003) developed a DGGE method to examine the diversity of the major capsid protein of *E. huxleyi*–specific viruses and demonstrated that only a few members of the *E. huxleyi* virus community actually caused the demise of a bloom observed in a mesocosm experiment. This observation was made again 3 years later at the same study site (Martinez et al. 2007).

Although the use of DGGE/PFGE to directly study aquatic viruses is limited to a relatively small number of reports, several studies have used DGGE to link changes in host community composition to changes in virus abundance and/or virus diversity as inferred from PFGE analysis of virus genome sizes (e.g., Castberg et al. 2001, Goddard et al. 2005, Larsen et al. 2001, Riemann and Middelboe 2002, Simek et al. 2001, Van Hannen et al. 1999b, Weinbauer et al. 2007, Zhang et al. 2007). Together, the body of literature describing DGGE/PFGE-based investigations of aquatic virus communities demonstrates the importance of these methods for aquatic virologists and convincingly demonstrates that fingerprint-based investigations have had a profound impact on current understanding of virus community ecology.

Materials and procedures

An overview of the methods involved in fingerprinted aquatic virus communities is shown in Fig. 1. Because the two methods considered in this chapter (PFGE and DGGE) are very different, each will be considered independently. Because the analysis of the fingerprint patterns generated by each method does not differ, this section will conclude with a brief comment on fingerprint analysis. Furthermore, in this section we have provided the formulation for many of the reagents used for PFGE and DGGE; nevertheless, more detailed recipes for many of them (e.g., electrophoresis buffers) can be found in most molecular biology laboratory manuals (e.g., Ausubel et al. 2002, Sambrook et al. 1989).

PFGE—The viral concentrate used for PFGE analysis must be molded into plugs, followed by lysis of the virus particles to release their DNA. It is possible to run solution-based preparation of viral DNA for PFGE (Steward 2001); however, large DNA molecules (>100 kb) are extremely sensitive to mechanical shearing in aqueous solution (Bouchez and Camilleri 1997). The consensus is that lysis inside viral plugs prevents mechanical shearing of the DNA, resulting in more discrete PFGE bands. Intact viral genomes are then separated by size by PFGE. After separation, the banding pattern is visualized by staining with a fluorescent DNA stain. This banding pattern provides a visual record of the genome size distribution that can be used for qualitative and quantitative comparisons between samples.

Equipment and reagents:
- Pulsed field gel electrophoresis system. We recommend the CHEF-DR II (Bio-Rad);
- Casting stand, comes in 14 × 13 cm (small gel, 10 wells) or 21 × 14 cm (large gel, 15 wells) frame and platform;
- Combination comb holder;
- Combs, 1.5 mm thick;
- Plug molds (Bio-Rad), each well holds 80 μL;
- Screened caps (Bio-Rad);
- DNA molecular weight standards, e.g., lambda ladder (available in blocks that have to be sliced into smaller pieces before use) and 5-kb ladder (e.g., Bio-Rad);
- Dilution or storage buffer: SM buffer (0.1 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl, 0.005% (wt/vol) glycerin);
- Lysis buffer: must be freshly made (250 mM EDTA, pH 8.0, 1% SDS, 1 mg/mL Proteinase K); 5 mL lysis buffer is needed per sample;
- 1.5% PFGE-grade agarose (e.g., SeaKem GTG agarose; Cambrex) for preparing plugs dissolved in TE 10:1 (10 mM Tris, 1 mM EDTA, pH 8.0); the agarose can be stored at 4°C in between uses;
- Washing buffer: TE 10:1 (10 mM Tris, 1 mM EDTA, pH 8.0), 150 mL per sample;
- Storage buffer: TE 20:50 (20 mM Tris, 50 mM EDTA, pH 8.0), 20 mL per sample;
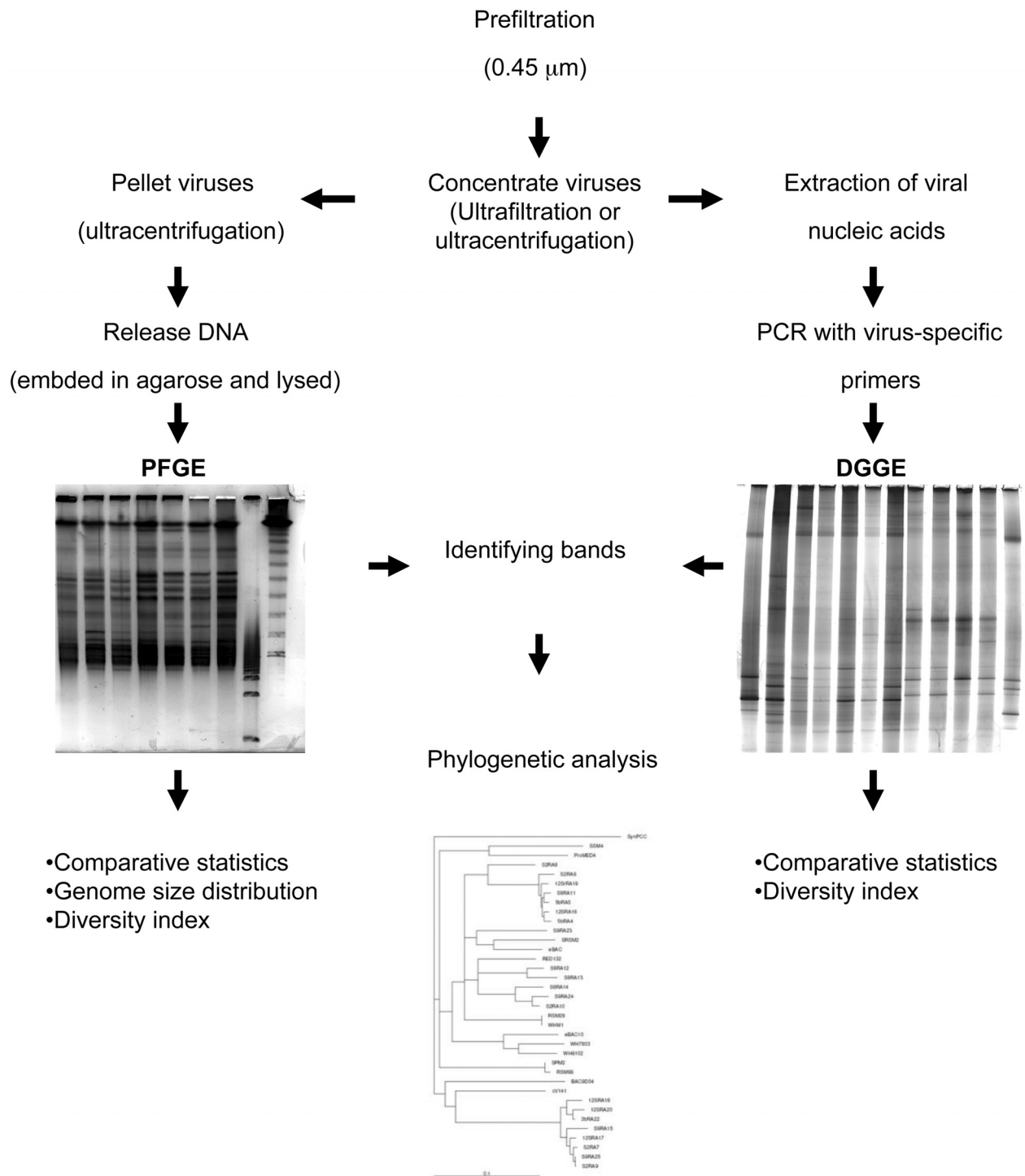
Prefiltration

(0.45 μm)

Pellet viruses

(ultracentrifugation)

Concentrate viruses
(Ultrafiltration or
ultracentrifugation)

Extraction of viral

nucleic acids

Release DNA

(embded in agarose and lysed)

PCR with virus-specific

primers

PFGE

Identifying bands

DGGE

Phylogenetic analysis

•Comparative statistics
•Genome size distribution
•Diversity index

•Comparative statistics
•Diversity index

Fig. 1. Flowchart of methods involved in fingerprinting aquatic virus communities.

- Running buffer: 2 L of 1 × TBE (10 × TBE stock: 108 g Tris, 55 g boric acid, 40 mL 0.5 M EDTA pH 8.0);
- Gel buffer: 1 × TBE, 100 mL (small gel) or 150 mL (large gel);
- 1% PFGE-grade agarose for gel electrophoresis (e.g., SeaKem GTG agarose; Cambrex);

- Fluorescent DNA strain, e.g., SYBR I or SYBR gold (Molecular probes);
- UV transilluminator and gel documentation system.

Molding of PFGE viral plugs: For long-term stability of viruses before PFGE and to avoid downstream interference

with nucleic acid extraction or electrophoretic properties of PFGE, we recommend buffering of the virus concentrate in SM buffer, prepared via either ultracentrifugation or dialysis (Wommack et al. 2010, this volume). Samples can be stored for 2–3 days in SM buffer, but we recommend you use it immediately. Each of the agarose plugs should represent the same amount of sample volume; alternatively, number of VLPs.

Prepare the 1.5% agarose and the lysis buffer. Incubate the agarose at 80°C until further use. Dispense 5 mL of the freshly made lysis buffer into 50-mL Falcon tubes, one for each sample. Combine equal volumes (200 μL) of virus concentrate and molten 1.5% agarose, mix briefly, and dispense the mixture into plug molds with a pipette. Avoid bubbles in the plugs. Place the plug molds in the freezer (–20°C) for at least 2 min to set. Remove the tape from the bottom of the plug molds and push the plugs out from the molds into 5 mL lysis buffer. Make sure that the entire plugs are submerged in the lysis solution. Incubate the plugs in lysis buffer overnight in the dark at 30°C.

The next day, decant the lysis buffer using a plastic sieve (screened cap) that can be attached at the top of the Falcon tube. Be sure that no plugs are stuck in the cap before moving on to decant the next sample. Wash the plugs three times, 30 min each, in TE buffer 10:1 at room temperature. The plugs can be stored at 4°C in TE 20:50 for several month before further processing; nevertheless, we recommend running the samples as soon as possible, because degradation of the viral DNA will occur and result in less discrete bands.

Gel preparation: Set up the gel rig. Be sure that the comb sits evenly along its entire length. Prepare a 1% agarose gel in 1× TBE buffer. Melt until the agarose is completely dissolved. Place the warm agarose at 60°C for 10 min before pouring into the gel rig and allowing it to cool. Avoid air bubbles in the gel. Keep ~5 mL agarose at 60°C for later use to seal the wells. When the agarose is set, pour 50 mL of 1× TBE running buffer on the top of the gel and place it in the refrigerator for at least 20 min or overnight. Place molecular weight standards (slices of ~5 mm) on either side of the gel. Place the samples between the markers using a sterile loop. Be sure that no air bubbles are trapped in the well. Overlay the wells with leftover molten 1% agarose. Remove the gel from the pouring rig and remove any extraneous agarose from the bottom and edges.

Electrophoresis: Prepare the 1× TBE (running buffer) and place at 14°C until further use. Place the gel into the electrophoresis chamber and carefully pour the cooled 1× TBE running buffer into the chamber. Run the gel at 6 V cm^{-1} with pulse ramps from 20 to 40 s (for example) at 14°C for 22 h. Size markers should be encompassed to facilitate size determination for all the different PFGE viral bands. A number of size markers for PFGE are commercially available. These conditions result in runs that make a good starting point for further analysis. To separate different viral genome size classes, each sample could be run three times: (1) 1–5 s switch time with 20 h run time for separation of small genome sizes (0–130 kb); (2)

8–30 s switch time with 20 h run time for separation of medium genome sizes (130–300 kb); (3) 20–40 s switch time with 22 h run time for separation of large genome sizes (300–600 kb). Stain the gel for 30 min or overnight in fluorescent stain (according to manufacturer's instructions) and view on a UV transilluminator.

DGGE—Using DGGE, similar-sized PCR products that differ in nucleotide composition (sequence) can be separated in denaturing gradient gels. Denaturing gradient gels are created using acrylamide (structural material) solutions that contain different amounts of denaturants (urea and formamide), such that the highest concentration of denaturants is at the bottom of the gel and the lowest concentration is at the top. As dsDNA fragments differing in sequence migrate into the gel during electrophoresis, they encounter increasing concentrations of denaturants, and each fragment partially melts (i.e., double-stranded regions dissociate into single-stranded) at a different place in the gel depending on its sequence. Because the electrophoretic mobility of partially melted DNA fragments is greatly reduced compared to dsDNA, same-sized DNA fragments with different sequences focus at different positions in these gels. Because PCR with universal primers can be used to amplify related, but different, DNA sequences, and different sequences focus at different positions in a denaturing gradient gel, DGGE can be used to produce a unique banding pattern, or fingerprint, for each PCR product amplified from different microbial communities.

Equipment and reagents:

- DGGE apparatus, e.g., Hoefer Scientific SE600, Bio-Rad DCode system (includes gradient former), Ingeny, CBS Scientific;
- Peristaltic pump and gradient maker for casting gradient gels, e.g., SG Gradient Maker (GE Healthcare);
- Power supply for electrophoresis systems;
- Denaturing gel solutions: Solution A (for 250 mL of an 8% gel solution with 0% denaturant), 50 mL 40% acrylamide/bis stock solution (37:5:1 acrylamide:bis-acrylamide solution), 2.5 mL 50× TAE, adjust to 250 mL with sterile distilled water (sdH$_2$O); and Solution B (for 250 mL of an 8% gel solution with 100% denaturant; i.e., 7 M urea and 40% formamide), 50 mL 40% acrylamide/bis stock solution (37:5:1 acrylamide:bis-acrylamide solution), 2.5 mL 50× TAE, 105 g urea, 100 mL deionized formamide, adjust to 250 mL with sdH$_2$O;
- 10% wt/vol ammonium persulfate solution in sdH$_2$O (APS); this reagent should be prepared fresh each time it is used;
- TEMED (N,N,N′,N′ -tetramethylenediamine);
- 1× TAE running buffer (from 50× buffer stock solution: 20 mM Tris-acetate (pH 7.4), 10 mM sodium acetate, 0.5 mM EDTA);
- 6× loading buffer (e.g., 60% glycerol, 10 mM Tris-HCl, pH 7.6, 60 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol FF);

- Fluorescent DNA strain (e.g., SYBR Gold or SYBR II gel stain; Molecular Probes);
- UV transilluminator and gel documentation system.

Gel preparation: Using lint-free tissues, wash glass plates, spacers, and combs thoroughly with 70% ethanol. Do not use soap or harsh abrasive cleaning materials to clean any of the equipment. If the materials are cleaned diligently, there is no need to use any detergents; a simple water rinsing followed by 70% ethanol will suffice. The following instructions will vary depending on the apparatus used; refer to the manufacturer for specific instruction pertaining to their system. Assemble the gel sandwich by placing the small glass plate on top of the large plate, being sure to correctly place a 1-mm spacer along each edge of the plate assembly. To prevent current leakage and the resultant "smiles" in the bands near the edges of the gel, grease both sides of the spacers with as little as possible silicon grease to cover the full length of the spacer but only a quarter of the spacer width. Attach the plate clamps and place the entire assembly into the casting stand. Inspect the plate assembly to ensure that the two glass plates and the spacers form a flush surface along the sides, and ensure that all gaskets adequately seal the plate assembly. Breaches in the seal of the plate assembly with the bottom of the pouring stand will result in leakage during gel polymerization. Check the gradient maker and flush with sdH$_2$O. Empty pump tubing and attach pipette tip at the outlet tube to the top-middle of the gel chamber. Although the reagents listed above specify the formulation of an 8% gel, the percentage of acrylamide in the gel depends on the size of the PCR products to be resolved; i.e., 6% gel is recommended for 300–1000 bp, 8% for 200–400 bp, and 10% for 100–300 bp (BioRad manual, DCode Universal Detection System).

To optimize the gradient conditions for a new DGGE experiment (new primer sets, new sample type/habitat, etc.), we usually start with a relatively broad gradient (20% to 80% denaturant). We then focus the gradient around the area of interest to include the highest and lowest bands in different samples. Table 1 can be used to determine the appropriate composition of the denaturing gradient gel (16 × 16 cm) that has a total volume of 29 mL. The size and volume may vary between the different apparatuses. Make up two solutions of 14.5 mL each, a low denaturant concentration solution and a high denaturant concentration solution. For example, if you wish to make a 30% to 55% gradient, then you would make a 30% (low) solution and a 55% (high) solution based on the reagent volumes in the table. Mix the solutions A and B to the desired percentage. Alternatively, once desired gradient conditions are empirically determined, each denaturing solution can be made directly using the reagent volumes and amounts shown in Table 2. After preparing the denaturing gel solutions, degas for 15 min and filter through a 0.45-mm syringe filter. Immediately before casting the gel, add 145 µL 10% APS and 7.25 µL TEMED into each solution and swirl gently to mix. These reagents begin the polymerization of the acrylamide. At this point, you will have approximately 10 min to pour the gel. We use the SG gradient maker attached to a peristaltic pump. In our hands, this system has produced more consistent gradients than other systems. Make sure the pump is off and the gradient maker-channel is closed (handle up). Pour solution with the highest denaturant in the right leg of the gradient maker (at the pump side) and the solution with the lowest denaturant in the left leg. Turn the magnetic stirrer on, while simultaneously starting the pump (5 mL/min). Simultaneously, start the pump (5 mL/min) and move the handle of the gradient maker to horizontal position (channel open). The gel chamber fills slowly. Use approximately 4–5 min to fill the gel. It is important to avoid bubbles in the gel, as this will stop the products from migration. Empty the tubing and flush thoroughly with sdH$_2$O. Insert the comb, flat or straight side down, making sure that there are no bubbles under the comb. This is to ensure a smooth, even finish when you come to create your wells after your gel has set. Different combs (16 or 20 wells) are available, depending on the number of samples that you want to run. Cover gels with cling film and allow ~2 h for the gel to polymerize. The gel can be kept at 4°C until the next day.

Electrophoresis: Different DGGE systems require different volumes of running buffer. The following procedure is based on the DCode system from Bio-Rad. Prepare approximately 7 L of 1× TAE and fill the buffer chamber. Put about 0.5 L aside for later use. To enhance the circulation of the running buffer, place the tank on a magnet stirrer and add a magnetic stirrer bar in the bottom of the tank. Preheat the buffer in the DCode apparatus to 60°C; this will take about 2 h. Attach the gel plates to the core assembly. Loosen the clamps a quarter-turn counterclockwise to prevent breaking of the sandwich clamps (due to heat expansion). Then place the core assembly into the heated buffer in the tank. Note, the following procedures can be carried out either while the gel is standing at the bench or when the core assembly is loaded in the tank. Switch off the magnetic stirrer (if loading in tank). Flush each well with buffer using syringe with needle to remove any unpolymerized acrylamide and excess urea. Failure to do this might result in uneven well floors and unresolved bands. Flush each well with buffer again before loading approximately 10–50 µL of PCR products mixed with loading dye into each well. The volume loaded depends on yield and the expected diversity of the PCR products. For quantitative comparisons among samples, equal quantities of DNA must be loaded in each lane. It is also recommended to use standard markers on the gels to allow gel-to-gel comparisons. The standard marker should be composed of fragments covering a range of denaturant concentrations. To quantify PCR products, we recommend gel quantification using a DNA mass standard (e.g., Low DNA Mass ladder; Invitrogen) and commercially available gel quantification software such as Quantity One (Bio-Rad) or free software such as Image J (available for download at http://rsbweb.nih.gov/ij/download.html). In the DGGE gel, load a marker on each side of the gel adjacent to the samples

Table 1. Formulation for DGGE gels using 0% and 100% denaturing solutions.

% denaturant of desired gel solution	Volume of solution A (0% denaturants)	Volume of solution B (100% denaturants)
0	14.50	0.00
15	12.32	2.18
20	11.60	2.90
25	10.87	3.63
30	10.15	4.35
40	8.70	5.80
45	7.97	6.53
50	7.25	7.25
55	6.54	7.96
60	5.80	8.70
65	5.07	9.43
70	4.35	10.15

Table 2. Denaturant amounts for various denaturing gel solutions.[a]

Denaturant concentration	10%	20%	30%	40%	50%	60%	70%	80%	90%
Formamide, mL	4	8	12	16	20	24	28	32	36
Urea, g	4.2	8.4	12.6	16.8	21.0	25.2	29.4	33.6	37.8

[a]For 8% acrylamide gels, add 20 mL of a 40% acrylamide/bis (37.5:1) solution, 2 mL 50× TAE, and bring the volume up to 100 mL using sdH$_2$O.

(markers can be custom made for each DGGE application using known PCR products, or common molecular weight markers can be used) for determination of band positions, or comparisons of different gels. Apply a loading voltage of 200 V for 5 min before starting the pump to circulate the buffer, then turn on the magnetic stirrer. The length of the run and the running voltage depend on the size of the PCR products and the percentage of acrylamide/bis in the gel. A good starting point is to run the gel at 60 V (about 20 mA for one gel) for 19 h. Optimal run times and conditions, however, should be empirically determined for each type of fragment.

When the electrophoresis is complete, take apart the apparatus and remove the glass plates from the gel clamps. Carefully separate the plates, leaving the gel exposed on the large plate. Use the edge of the small plate to trim the well walls, but be sure to leave the leftmost wall slightly higher than the others for use as a gel orientation reference. For easy manipulation, the gel can either be stained on the large plate or transferred to, stained on, and transported on a plastic sheet. Stain the gel for 30 min in 50–500 mL fluorescent gel stain (depending on the container, and according to manufacturer's instructions). Destain the gel for 30 min in 1× TAE (not always necessary). Remember, the fluorescent dye binds to nucleic acids; therefore, it is important to minimize contact with skin, so gloves (powder-free) should be worn. If staining in a container, use plastic and not glass, as the fluorescent dyes accumulate over time on glass surfaces. Slide the gel off of the plastic sheet or large plate onto a UV transilluminator and view the gel. As an alternative to staining gels with fluorescent dyes, some researchers have used fluorescently labeled PCR primers for DGGE analyses (Neufeld and Mohn 2005).

Analysis of PFGE and DGGE fingerprints—DGGE and PFGE fingerprints can be analyzed using a variety of commercially available gel analysis software products (e.g., GelCompar II, Applied Maths; BioNumerics 5.1, Applied Maths; Quantity One, Bio-Rad). A common method to analyze DGGE/PFGE fingerprints involves creating a binary matrix representing the bands occurring in a set of DGGE/PFGE patterns. The presence or absence of bands in a sample is simply scored in a binary manner as 1 (present) or 0 (absent), relative to all of the bands detected in a set of DGGE/PFGE patterns. The binary data can then be presented in a dendrogram where the differences in fingerprint patters are represented in a graphical format or as a dendrogram using a distance-based cluster analysis techniques such as unweighted pairwise grouping with mathematical averages (UPGMA). Another possibility is to use multidimensional scaling (MDS) to reduce a complex fingerprint pattern to a point in a two-dimensional space (Van Hannen et al. 1999a). It is important to note that these types of analyses depend on consistency when detecting bands, and subjective determination of presence or absence should be avoided. Luckily, most commercially available gel analysis software programs allow researchers to use automated band detection parameters (e.g., band width and intensity), or even set their own thresholds for each parameter. As an alternative to comparisons based on presence or absence, the overall pattern of gel lanes can be compared directly using densitometry profiles (i.e., the pixel intensities at discrete positions in the gel). This type of analysis is based on pairwise correlations of profiles and can be used to avoid biases associated with band detection

or loading unequal amounts of DNA. For densitometry profile analyses, each lane profile is compared to the others, and the resulting matrix of correlation values correspond to lane similarities. The correlation values can then be transformed to dissimilarity values (1 – similarity) that can be used for cluster analysis via UPGMA.

Quantitative analysis of DGGE and PFGE gels is also possible. For DGGE, only semiquantitative analysis is possible, since individual band intensities cannot be used to infer target abundances in natural samples because differences in band intensity can arise from variable amplification efficiencies for different targets, and/or differences in background DNA. Thus fingerprint patterns can be compared, but individual band intensities should not be used to infer target abundance in natural samples. For PFGE, quantitative analysis involves measuring the relative fluorescence of each band. Based on this information, it is possible to obtain values for richness and abundance that can be used to calculate diversity indices. This type of analysis can be conducted using the commercial software noted above. Of course, it should be noted that both DGGE and PFGE are subject to a number of confounding errors (see "Assessment"). These sources of error should be carefully considered when deciding what types of analyses or comparisons should be conducted and not the least when the outcome of the analysis is interpreted.

Assessment

The PFGE fingerprinting technique provides a PCR-based independent approach that can be used to characterize viral assemblages from diverse environments. The technique is capable of electrophoretic separation of DNA fragments ranging from <10 to >1000 kb in length, by applying a pulsed electric field with alternating orientation (Birren and Lai 1993, 1994). The pulsed electrical field forces pieces of DNA to move in different directions. Although the predominant direction is toward the bottom of the gel, the back-and-forth motion allows the large DNA fragments to migrate their way through the gel. Thus, PFGE analysis can differentiate between the many different viral genome sizes found in the aquatic environments, such as sizes ranging from the giant virus genomes belonging to the family Phycodnaviridae (>150 kb) to the smaller single-stranded DNA chp1-like microphage (<10 kb). As this technique makes use of an extraction and separation method that places minimal shear on the genomes, the result of the analysis is a banding pattern that provides a visual record of the genome size distribution within a given sample. In practice, from a single sample 10–30 different bands (viral populations) can be resolved. In the marine environment, it has been shown that the most abundant viral populations are those with the smallest genomes, between 20 and 80 kb; less abundant are viruses with middle-sized genomes, between 80 and 280 kb, and viruses with the largest genomes, between 280 and 500 kb, are the least abundant group (Sandaa 2008). In general, the distribution of environmental genome size cor-

responds with the genomic size range of viruses in culture and the abundance of the respective host community in the marine environment (Sandaa 2008, Steward et al. 2000).

The PFGE assay will detect only the most abundant viral populations in the sample. In mesocosm studies, Sandaa and colleagues have been able to detect between 5 to 11% of the total viral community using PFGE. The lowest detectable virus number was approximately 10^2 particles mL^{-1} and was found for a viral population with a genome size of 487 kb (R.-A. Sandaa, unpublished results). The first critical step of the PFGE analysis is to obtain a high-titer viral concentrate (Wommack et al. 2010, this volume). This concentration step is important for the sensitivity of the assay, and all biases in this step will be reflected in the PFGE analysis. The sample volume required can vary greatly depending on the initial concentration of viruses. Nevertheless, there will be a lower threshold level were the bands no longer can be detected on the gel. The intensity of a given band on a PFGE gel will in addition depend on the size of the virus genome. It follows from this that a virus population with a genome size of 19 kb have to be in a concentration of 2.6×10^3 particles mL^{-1} to yield the same band intensity as a virus population with a genome size of 487 kb and 10^2 particles mL^{-1}. Quantitative analysis of banding patterns are possible; however, several factors should be kept in mind when analyzing these gels. Different viruses might have the same genome size and therefore be indistinguishable on a PFGE gel. This means that the band number is a minimum estimate of the total viral diversity. In addition, there is some difficulty in resolving different viral genomes within narrow size ranges. This might occur as a diffuse band on the gel. To reduce the latter uncertainty, each sample can be run several times, with focus on separation in different genome size ranges (for more details about limitations of PFGE for viral community studies, see Steward 2001). Another concern that should be considered is that some viruses do harbor several genomes of different sizes; thus one band does not necessarily mean one viral population (Holmfeldt et al. 2007). Taking all the limitations of the method into consideration, PFGE might not catch all shifts in the viral community; however, the method has been proven to be useful for observing the dynamics of viral communities in a variety of aquatic environments (Riemann and Middelboe 2002, Sandaa and Larsen 2006, Steward et al. 2000, Wommack et al. 1999).

DGGE separates PCR-generated DNA fragments by using different sets of oligonucleotide primers designed to target different genes. PCR of environmental DNA can generate amplicons with different DNA sequences that represent many genotypes. Because PCR products from a given reaction are of similar size (bp), however, conventional separation by agarose gel electrophoresis results in only a single DNA band that does not provide any indication of the number of different sequences that were amplified. DGGE can overcome this limitation by separating PCR products with different sequences based on the differential denaturing characteristics of the indi-

vidual DNA fragments. The technique exploits (among other factors) the different stability of G–C pairing (three hydrogen bonds per pairing) as opposed to A–T pairing (two hydrogen bonds). A mixture of DNA fragments of different sequence can be separated in an acrylamide gel containing a gradient of increasing DNA denaturants. In general, DNA fragments richer in GC will be more stable and remain double-stranded at higher denaturant concentrations than fragments with lower GC content. Because double-stranded DNA fragments migrate faster in acrylamide gels than partially denatured molecules, partially denatured molecules are effectively larger, and their electrophoretic mobility is greatly reduced. Thus, DNA fragments of differing sequence (i.e., G + C content) can be separated in an acrylamide gel containing a linear gradient of chemical denaturants. Theoretically, each band in a DGGE gel represents a different microbial population present in the community, and therefore, each PCR of an environmental sample can produce a unique fingerprint that reflects the composition of the community amplified. Hence, DGGE fingerprints can be used to compare the virus community composition of different aquatic environments.

In addition, the increased stability of G–C pairing can be exploited to increase the resolution of DGGE. Early in the development of DGGE, it was demonstrated that attachment of a 40-base-pair G + C–rich sequence (i.e., GC-clamp) to one end of PCR fragments prevented complete denaturation of short amplicons and provided better resolution of very similar sequences. Attachment of GC-clamps to PCR fragments is easily achieved by adding a GC-clamp to the 5′ end of one of the PCR primers (Sheffield et al. 1989, 1992). An important caveat for the use of GC-clamp primers is that they can compromise the efficacy of the original primer set. However, this problem can be easily overcome by conducting two stages of PCR. During this two-stage procedure, the first round of PCR is conducted with the original primers to amplify specific DNA fragments from natural samples, and the second round is conducted using first-round amplicons as templates (e.g., gel-purified PCR products) and a modified GC-clamp primer.

Like any molecular technology, DGGE is subject to certain specific limitations, and researchers using this technique should be aware of these limitations and interpret their DGGE fingerprints with caution. Perhaps the most significant caveat researchers should be aware of when using DGGE is that it is possible that some bands appearing in a fingerprint are the result of amplification or electrophoretic artifacts. Obviously, the inclusion of artifact bands in community analyses could confound similarity comparisons and lead to erroneous conclusions. Documented examples of these artifacts have been published. For example, Janse et al. (2003) used DGGE to differentiate different strains of cyanobacteria by amplifying the 16S to 23S internal transcribed spacer (ITS) region of rRNA genes. Although these workers were able to discriminate closely related cyanobacterial strains using this DGGE method, they also noted that ITS rDNA amplification from

several strains produced multiple bands in DGGE gels. In their report, they concluded that some cyanobacteria may have more than one rRNA-ITS operon, and cautioned that the presence of multiple operons in a single organism, or PCR (e.g., chimera and heteroduplex formation) and electrophoretic artifacts (e.g., comigration of different DNA fragments), could confound studies of complex microbial consortia (Janse et al. 2003). Eventually, these same researchers demonstrated that increasing the final elongation step in their PCR helped alleviate artifacts arising from the production of multiple bands from a single target molecule (Janse et al. 2004). It should be noted that previous studies have also documented the comigration of different DNA fragments in DGGE gels (e.g., Sekiguchi et al. 2001), and that band re-amplification and sequencing from very different regions in a DGGE gel can produce identical sequences (Nikolausz et al. 2005). Based on these observations, Nikolausz et al. (2005) concluded that "the banding pattern in a DGGE gel may not be simply the result of the separation of different amplicons according to their melting behavior, but a consequence of complex interactions among different DNA structures." Thus, it is important to consider these potential artifacts when interpreting DGGE-based community analyses.

Discussion

That there is no single universal gene present in all viruses has made it difficult to make inferences regarding the total viral diversity in natural viral communities. However, genome size is a universal phenotypic characteristic of sufficient variability to be used as a proxy for classification of the most abundant populations of dsDNA viruses. PFGE provides a whole-genome fingerprinting of the most abundant and probably most active viral populations in a sample. This information can be used to reveal variability in the viral composition over space and time. In addition, the availability of quantitative analysis of viruses belonging to the different genome size populations makes it possible to use the assay for diversity studies (Larsen et al. 2004, Sandaa et al. 2003). Further, as the method is based on intact viral genomes, it is possible to identify any band of interest by, for instance, specific viral primer or whole-genome sequencing (Sandaa et al. 2008, Santos et al. 2007). PFGE can thus serve as a starting point for more detailed studies of the viral community composition and can be used to study the ecology of important viral groups in the ocean without the need of a cultivable host.

Despite the fact that there are inherent limitations associated with DGGE, the positive aspects of the technique (it is fast, inexpensive, and relatively simple to use and the results are reproducible) outweigh its limitations. Although identical or nearly identical DGGE fingerprints can be produced from the amplification of different targets, different fingerprints cannot be produced from the same target given identical experimental conditions. In a similar way, PCR is a "unidirectional" method that permits the conclusion that a target is

present, but not that it is absent from a sample; obviously, this constraint on PCR-based experiments has not limited its widespread use throughout the life sciences! Thus, DGGE should be considered an effective tool that, for example, can be used to guide other components of a research program. As just one example of the many potential uses of DGGE, the method can be used to focus clone library construction and sequencing efforts on samples that produce different fingerprint patterns. In this way, the recovery of different sequence types can be maximized while circumventing the need to clone and sequence every sample in an experimental series. Given an appreciation for its limitations and pitfalls, DGGE remains an extremely useful tool for studies of the molecular ecology of aquatic virus communities.

Comments and recommendations

Although PFGE and DGGE each have their own specific limitations, they have been widely used to study aquatic microbial ecology. Because PFGE is based on the analysis of intact virus genomes, it can be used to provide a snapshot, or fingerprint, of the richness and dynamics of entire aquatic virus communities. DGGE, on the other hand, is based on the analysis of PCR-amplified gene fragments and can be used to fingerprint the richness and dynamics of specific groups of aquatic viruses. As noted above, both of these methods have definite pitfalls, and researchers are encouraged to be cautious when interpreting their fingerprint data. Nonetheless, after an initial setup cost for the equipment, both of these methods are relatively inexpensive, quick, and easy, and the results are reproducible. Thus, it is likely that these methods will continue to be useful tools for the study of aquatic viruses for years to come.

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