MANUAL
of
AQUATIC VIRAL ECOLOGY

MAVE Chapter 18, 2010, 182–192
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Basic electron microscopy of aquatic viruses

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Abstract

For identification and structural studies, viruses are concentrated and purified by differential or density gradient centrifugation, stained with phosphotungstate (PT) or uranyl acetate (UA), and examined by transmission electron microscopy. Both PT and UA produce artifacts. PT yields negative staining only; UA produces both negative and positive staining and often gives excellent contrast. Fixation is normally not necessary. UA-stained preparations can be stored for years. Virus particles may be sedimented directly from water samples onto grids by means of special centrifuge tubes and subsequent staining. Positively stained particles are highly contrasted and easy to count at low magnification. Positive staining also provides information on bacteria containing viral particles and rough estimates of burst sizes in individual bacteria. Isometric, filamentous, and pleomorphic viruses are identified after negative staining.

The domain of aquatic viruses includes bacterial, archaeal, and algal viruses. Bacterial viruses, commonly known as phages or bacteriophages, include cyanophages or viruses of cyanobacteria, formerly referred to as "viruses of blue-green algae." Bacteriophages were discovered in 1915 by Frederick William Twort and, independently, in 1917 by Felix d'Hérelle (Ackermann 2008). The first cyanophage, a "phycovirus" infecting *Phormidium,* was described in 1964 (Safferman and Morris), followed in 1974 by the first true algal virus, a virus of the brown alga *Ectocarpus* (Clitheroe and Evans) and the first archaeal viruses, two-tailed *Halobacterium* phages (Torsvik and Dundas). In 1991, 44 algal virus taxa were known to contain or produce viruslike particles (Van Etten et al.). Unfortunately, no recent review of this interesting subject is available.

Indigenous marine phages were found as early as 1960 (Spencer), but it was realized rather belatedly that marine phages, including cyanophages, were ubiquitous and occurred in enormous numbers in nature. It appears now that bacteriophages are the most abundant life form on Earth, their global population being estimated at 10^{31} viruses (Hendrix 2003). The frequency of eukaryotic algal viruses can only be guessed, and their classification is still at its beginnings. So far, only algal viruses of the *Phycodnaviridae* and *Marnaviridae* families have been officially classified by the International Committee on Taxonomy of Viruses (ICTV) (Fauquet et al. 2005). It is clear that many more algal viruses occur in nature and await classification. In addition, seawater contains unusual and novel entities such as mimiviruses (La Scola et al. 2003, Suttle 2005).

The ICTV classifies viruses primarily by nucleic acid and physicochemical properties of the virion, but any viral property may be used for classification. Viruses contain dsDNA, ssDNA, dsRNA, or ssRNA and are isometric, helical, pleomorphic, or tailed. They are presently categorized into three orders, 67 families, and 282 genera (Fauquet et al. 2005). New viruses are continuously described. Virus classification is a long and stepwise procedure. Bacterial and archaeal viruses are grouped together as prokaryote viruses. More than 5500 prokaryote viruses have been examined in the electron microscope (Ackermann 2007), with less than 300 of them being fully sequenced in 2007 (Ackermann and Kropinski). This suggests that we are still at the thresholds of viral ecology and that new discoveries are waiting to be made anytime. Approximately 96% of prokaryote viruses are tailed. They belong to the *Myoviridae*, *Siphoviridae*, and *Podoviridae* families (Ackermann 2007; Ackermann and DuBow 1987; Fauquet et al. 2005) and are easily identified in the electron microscope. Iso-

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Acknowledgments

Publication costs for the Manual of Aquatic Viral Ecology were provided by the Gordon and Betty Moore Foundation. This document is based on work partially supported by the U.S. National Science Foundation (NSF) to the Scientific Committee for Oceanographic Research under Grant OCE-0608600. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NSF.

ISBN 978-0-9845591-0-7, DOI 10.4319/mave.2010.978-0-9845591-0-7.182

Suggested citation format: Ackermann, H.-W., and M. Heldal. 2010. Basic electron microscopy of aquatic viruses, p. 182–192. *In* S. W. Wilhelm, M. G. Weinbauer, and C. A. Suttle [eds.], Manual of Aquatic Viral Ecology. ASLO.

metric, filamentous, and pleomorphic viruses are much more difficult to recognize. Algal viruses are isometric or filamentous. The major hosts of marine phages are proteobacteria of the genera *Pseudomonas* and *Vibrio* and cyanobacteria of the genera *Synechococcus* and *Prochlorococcus*.

Electron microscopy was introduced in the 1930s. It is a research field on its own and a basic technique of microbiology. The first virus images were published in 1938 and showed the mouse ectromelia virus, a poxvirus (von Borries et al.). The first bacteriophages, mostly coliphages with short tails, were observed in 1940 (Pfankuch and Kausche; Ruska). As early as 1943, a morphological classification of all viruses was proposed (Ruska). Viruses were first examined unstained and later after shadowing with a metal to enhance contrast. Shadowing, a cumbersome and aggressive procedure, was long ago replaced by negative and positive staining. Presently, the principal technique in use is the examination of negatively stained particles in transmission electron microscopes. The introduction of digital electron microscopes and digital cameras has created a whole new situation with occasionally unfortunate results (*see* below).

The challenges in the electron microscopy of aquatic viruses are still numerous. How do we identify a virus among other particles in, for example, the marine environment? Why do we hardly recognize filamentous viruses in these samples? Would more detailed studies of negatively stained preparations add to our knowledge of viral impact on microbial ecosystems? How to circumvent the use of uranyl acetate, as it is slightly radioactive and its use is restricted in many countries? The objective of this article is to summarize the general methods used for morphological studies, total virus counts, and other applications of electron microscopy in an ecological context. Our ultimate aim is to improve the quality of environmental electron microscopy.

Materials and procedures

1. General—

Virological electron microscopy has essentially descriptive purposes, namely the identification and characterization of free viruses. Of the many biological applications of electron microscopy (EM), only transmission electron microscopy (TEM) is of significant importance in virology. Its other applications, e.g., scanning electron microscopy (SEM), cryo-EM with or without three-dimensional image reconstruction, immuno-EM, sectioning of infected cells, shadowing of individual particles, or enzymatic virus digestion on grids, have limited applications and are sometimes practiced in a few specialized laboratories only. In aquatic virology, sectioning is still important because it permits identification of intracellular assembly sites of novel viruses and the detection, in mixed host systems, of which host cell produces viruses. An excellent overview of sectioning techniques may be found in a recent book (Kuo 2007). The direct harvesting of particles onto grids for total viruslike counts was and is of great importance for the understanding of viral activity in aquatic ecosystems (Bergh et al. 1989).

Negative staining was introduced in 1959 (Brenner and Horne) and revolutionized virology in generating virus images of unprecedented clarity. It is indispensable for structural studies and virus identification, but is also used for particle counts (below). The principle of negative staining is to mix the particles to be examined with an electron-dense solution of a metal salt of high molecular weight and small molecular size. On an electron microscopic grid, the particles then appear white or gray on a dark background. Many variant procedures have been devised. A detailed description of stains and procedures may be found elsewhere (Ackermann and Dubow 1987; Hayat and Miller 1990; Kay 1965).

2. Specific techniques—

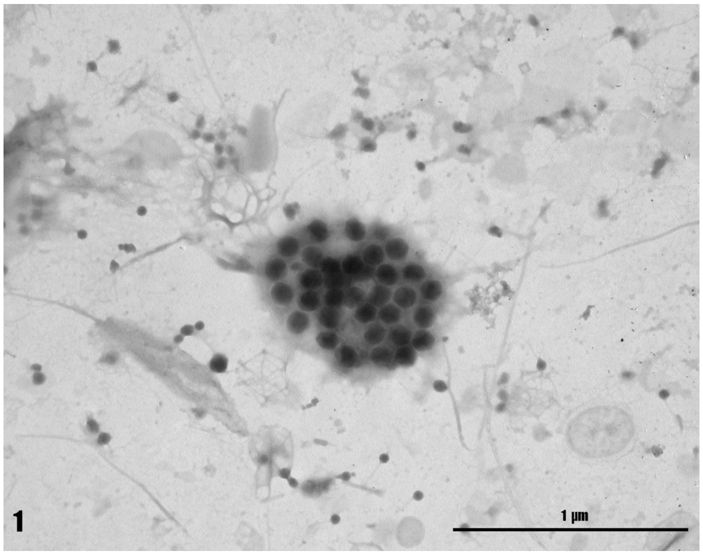
a. Purification:

For high-resolution images, samples should be sterile and free from bacteria and other large particles. This is achieved by filtration through membrane filters of 0.22–0.45 µm pore size. Except for particle counts on EM grids (below), purification is a must because proteins and slime interfere with staining and resolution and their amounts must be reduced to acceptable levels. The most convenient procedure is washing in a buffer, e.g., 0.1 M ammonium acetate (pH 7), in an ultracentrifuge with a fixed-angle rotor (25,000*g* or 25,000 rpm for 1 h). Centrifugation in swinging-bucket rotors requires higher *g* forces and centrifugation times (e.g., 70,000–80,000*g* for 60–90 min). Clearly, fixed-angle rotors are advantageous as they permit the use of relatively inexpensive medium-size centrifuges. Phosphate buffer is acceptable, but physiological saline is not because it leads to the formation of salt crystals on grids. Alternatively, viruses may be purified by CsCl or sucrose gradient centrifugation followed by dialysis.

b. Particle counts on EM grids:

Viruses in lysates are counted by TEM after depositing or vaporizing a fixed volume of virus suspension on the grid (Figs. 1 and 2). Viruslike particles (VLPs) in water can be counted by TEM, flow cytometry, or epifluorescence microscopy (EFM). The direct harvesting of particles on TEM grids ensures that bacteria, algae, and other particles can be observed simultaneously. These preparations can also be used for estimating bacterial size distribution and the frequency of infected cells and dividing bacteria, and to visualize lysed bacteria.

The technique requires an ultracentrifuge with a swinging-bucket rotor and centrifugation tubes (Polyallomer®) with flat bottoms, created by introducing Epoxy resin into a conical tube. The resin is left to harden at 60°C overnight, and an electron microscopic grid is deposited on the flat resin surface. Samples are centrifuged at 80,000*g* for 90 min (Borsheim et al. 1990). Tubes fitting Beckman SW-41 rotors allow particle harvesting from a 60-mm water column. Use cellulose nitrate filters fitting the diameter of the tubes, add a small piece of double-sided tape, and position the grids at the edge of the tape in the middle of the tube. Fill the tubes with sample and then add the filter paper with the grids attached to avoid air bubbles being trapped under the grids. The tubes should always be



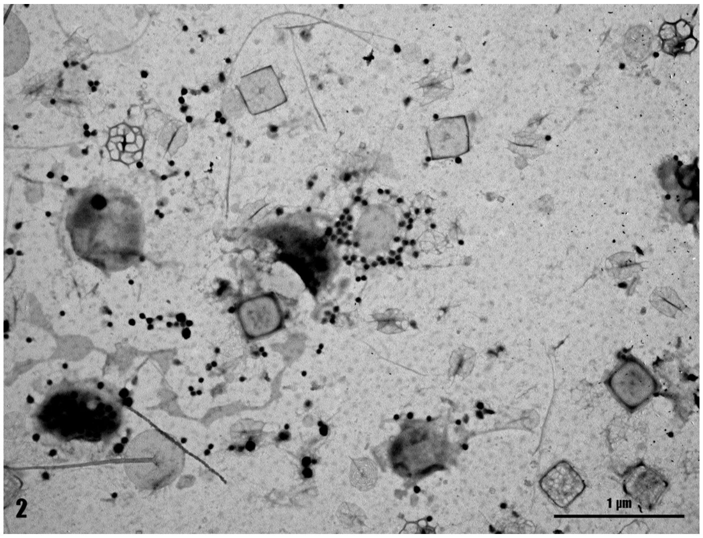


Fig. 2. Viral cluster outside a lysed cell.

Figs. 1 and 2. Particles harvested by centrifugation onto electron microscopic grids. Samples from Raunefjord near Bergen were sedimented onto 400-mesh Ni grids carrying carbon-coated Formvar films, using a Beckman Coulter ultracentrifuge, an SW-41 rotor, and Polyallomer tubes with molded flat bottoms. Grids were stained for 1 min with 2% UA, air-dried, rinsed in distilled water, and examined in a JEOL TEM 100.

Fig. 1. Marine bacterium with mature phages before lysis and free phages in the vicinity. Note the size variation in viruslike particles. The squares and crownlike structures are scales of algae, e.g., *Pyramimonas orientalis* (Chlorophyta).

filled up to 2–3 mm from the top. If particle density is high (10^7–10^8 cells mL^{-1}), the sample volume can be reduced. This can be done by reducing the height of tubes. The calculation of total virus contents is described by Bratbak and Heldal (1993) (*see* Appendix).

Montanié et al. (2002) presented a different approach to total virus counts by TEM. A purification step is followed by pelleting viruses from the supernatant by means of an ultracentrifuge. The particles are then transferred by capillarity to grids with carbon–collodium membranes. The recovery of viruses is reported to be 71% to 79%. The filtration technique uses membrane filters of 0.2 µm pore size for virus concentration. Virus particles on the filter are then resuspended in water and the suspension is examined in the EM (Torrella and Morita 1979). Again, this technique is inexact because particles smaller than 0.2 µm may be lost and also because of uncertainties introduced by resuspension. The technique of Ewert and Paynter (1980) involves sedimentation of viruses by means of a special rotor onto agar blocks and the preparation of pseudoreplicas, which are then stained and examined in the EM. The technique is quite complicated and has, to our knowledge, not been used by anybody else. We have no personal experience with it.

c. Special applications:

TEM has been used to visualize viruses within infected, unstained cells (Fig. 1). The aim is to determine burst sizes, lytic cycle lengths, percentages of bacteria in a stage of lytic infection, and mortality rates. The subject has been aptly reviewed by Weinbauer (2004).

d. Stains:

The stains used are tungstates (potassium, sodium, lithium, silicotungstates), uranyl salts (acetate, formate, magnesium acetate, nitrate, oxalate), molybdic acid, and ammonium or vanadium molybdates. The most common stains are aqueous solutions of sodium or potassium phosphotungstate (PT; usually 2% and pH 7.2) and uranyl acetate (UA; 1%–2%, pH 4–4.5). Stains are prepared by dissolving phosphotungstic acid or uranyl acetate in distilled water and adjusting the pH with KOH or NaOH. They can be kept in stoppered bottles at 4°C for 2 years. Uranyl salts have a strong affinity to double-stranded DNA (Huxley and Zubay 1961), are toxic and radioactive, and should be handled with care. PT produces negative staining only. UA produces negative staining and, because of its affinity to dsDNA, will stain positively in black any particles with sufficient dsDNA content, for example, phage heads or herpesvirus capsids.

e. Staining:

The required supplies include pointed pipettes and tweezers, strips of filter paper, and common electron microscopic grids (Athene-type, copper or steel, 100–400 mesh or square holes). Grids must carry a Formvar or collodium film stabilized by a carbon layer. They may be prepared by the electron microscopist or purchased ready-made. After 1 month, grids tend to be hydrophobic and reject particles. Grids can be made hydrophilic again by glow discharge in a carbon evaporator or by rinsing with a wetting agent (Alcian blue, bacitracin, poly-L-lysine, serum albumin, sucrose) (Gentile and Gelderblom 2005; Gregory and Pirie 1973). One of the authors (H.-W. Ackermann) sometimes uses UA or PT supplemented with 2 drops/mL of a 0.5% bacitracin solution.

The staining procedure is extremely simple and near-instantaneous. A drop of virus suspension is deposited on a grid, left 30 s for adsorption of viruses, and then a drop of stain is added, and the liquid is withdrawn with filter paper. Positive staining takes a little longer, but is complete after 1 min. Preparations prepared for TEM by centrifugation are stained with 1%–2% UA in water for 2–3 min, gently washed in distilled water, and air-dried. This will give a positive staining which facilitates total virus counts and an estimation of the number of infected cells and burst sizes (*see* Weinbauer 2004 and references therein). Positively stained viruses appear as electron-dense and deep black. The presence of impurities may create a high background noise.

f. Electron microscopes and imaging:

Transmission electron microscopes (TEMs) are operated at 60–80 kV. To limit contamination by hydrocarbon molecules present in the column and hence loss of resolution, the object stage may be cooled with liquid nitrogen. This is not necessary for experienced users. Three types of instruments are presently in use.

(1) Conventional TEMs. Lens columns, astigmatism, focusing, and magnification are adjusted by the user. Images are recorded on films or plates and are as much a product of the darkroom as of the EM. They require a darkroom equipped with a enlarger of professional quality. Mercury lamps and exhausts are not necessary, but small table-top enlargers as used by amateur photographers are inadequate. Supplies include fine-grain films, high-speed developers, polycontrast paper, and graded gelatin filters for enhancing contrast. Image contrast generally depends on developers, and papers and can be dramatically improved by filters. It is unfortunate that some photographical supplies, especially papers, have become difficult to find or have even disappeared from the market following the introduction of digital cameras.

(2) Automated TEMs. Images are recorded as above on film or plates, but focusing is automated, columns are largely self-contained, and resolution and magnification can no longer (or only with great difficulty) be adjusted by the electron microscopist. Their operation has thus been considerably simplified, but the operator has little control over the instrument and depends largely on the installer for alignment, resolution, and magnification control. Most of these instruments are now equipped with digital cameras and are commonly called "digital electron microscopes."

(3) Digital (charged-couple device [CCD]) cameras. The cameras are not made by EM manufacturers and must be purchased separately. Images are recorded electronically and are easy to store, retrieve, copy, and exchange. A darkroom is no longer necessary, but images must be printed on special paper and by means of special printers.

If no TEM is available, it is possible to use a scanning electron microscope (SEM) equipped with a field emission gun (FESEM) and scanning–transmission electron microscopy (STEM) detector. Most of these instruments can be run in STEM mode and used as ordinary TEMs. Preparation of samples and staining are as described for TEM. FESEM instruments are normally operated at 10–40 kV, but for magnifications up to ×150,000, reasonably good total counts of viruslike particles are obtained. One example is the JEOL instrument JSM-7400F (cold emitter), with which unfixed and unstained marine viruses were observed in STEM mode at a magnification of ×100,000 (M. Heldal, unpubl. data).

The other advantage of FESEMs is the possibility of obtaining high-resolution images in scanning mode, even at low accelerating voltages (1.5–2.5 kV). In such cases, the evaporation of gold/palladium on the material may give a coarse metal surface, and evaporation of iridium should be preferred. As an example, a Zeiss SUPRA 55 VP instrument (FESEM) equipped with an in-lens detector can be run at enlargements of up to ×500,000. Altogether, the new-generation SEMs (FESEMs) may be a good choice for providing good resolution microscopy for both TEM and SEM preparations. A drawback is the time-consuming setup of the STEM detector.

g. Magnification control:

High magnification (×250,000 or more) is controlled by means of grids with beef liver catalase crystals (Luftig 1967) or T4 bacteriophage tails. Diffraction grating replicas are suitable for low magnification only (below ×30,000). Calibration grids can be prepared in advance and kept for at least 1 month. Catalase crystals have parallel lines with 8.8-nm periodicity, can be used after PT and UA staining, and are commercially available. At a magnification of ×300,000, 20 lines correspond to 5.2 cm. T4 phage tails are 114 nm long, which corresponds to 34 mm at the same magnification. In conventional electron microscopes, magnification should be monitored often because of possible fluctuations in the local electricity supply. This is easily done by taking a picture of a calibration grid every 20–50 photographs and adjusting the darkroom enlarger. Digital electron microscopes and cameras are calibrated by the supplier or installer. This is a difficult procedure; it is doubtful whether these adjustments are all done by competent operators and it is so far unclear how stable digital cameras are over long periods.

3. Assessment—

a. Purification:

Centrifugation does not eliminate bacterial debris (cell wall fragments, flagella, pili, capsule material), but this is not a real problem as these particles do not interfere with resolution and it is always possible to find areas without them. In addition, almost all centrifuged preparations contain phage debris (e.g., isolated heads or tails) and abnormal or defective particles (polyheads or polytails). Using a fixed-angle rotor, a force of 25,000*g* is amply sufficient for tailed phages, the largest of which sediment at 6000*g* for 1 h. It is just sufficient for very small viruses, e.g., microviruses or filamentous phages, which require 30,000*g* for 1 h for complete sedimentation (Ackermann and Dubow 1987). The technique described above is akin to centrifugation in the Beckman Coulter Airfuge which, however, uses extremely small volumes (180–240 µL) and is thus unsuitable for environmental studies (*see* above, 2a). Density gradient purification is not mandatory for environmental studies. Such preparations can be very rich and pure, but some are full of crystals and impurities. The technique critically depends on adequate dialysis, demands large amounts of material, and can be recommended only if the performing scientist is sure of his or her skills.

b. Particle counts and special applications:

Counting of viruses in lysates can be difficult because viruses often do not disperse well or adhere poorly to the grid. For example, phages may aggregate with each other or cellular debris and even separate into areas of intact and damaged particles. Both problems can be overcome by washing grids with a solution of poly-L-lysine (Müller et al. 1980). Visualizing viruses within unstained infected bacteria is problematic because of bacterial inclusion bodies, notably carboxysomes (Shively et al. 1973) and polyhydroxybutyrate (Tian et al. 2005). Carboxysomes, which occur among others in the important cyanobacterium *Synechococcus,* resemble phycodnaviruses in diameter and hexagonal shape, but are recognizable by their variable size and distinctive capsomerlike structures.

c. Stains and staining:

Phosphotungstate (Table 1) is a neutral stain and produces negative staining only. Artifacts are few and results are predictable and constant. Full phage heads often appear rounded in PT; empty heads appear as membranes or are filled by stain and then resemble black vesicles. Ammonium molybdate produces the same type of staining as PT and has no advantage over it.

UA is an acid stain and causes both negative and positive staining. It is unpredictable and there is no way to produce positive or negative staining consistently and reliably. Both types of staining are usually present on the same grid and even on adjacent parts of the same grid hole (Fig. 3). However, proteinic impurities seem to facilitate negative staining. Negatively stained phage heads are white or grayish when full, but appear as thick membranes when empty. Positively stained viruses (or phage heads) are deep black, indicating the presence of dsDNA. They can easily be detected at low magnification, which is advantageous in environmental studies (*see* Borsheim et al. 1990). Positively stained phage heads are invariably shrunken by 10% to 15% (Figs. 4 and 5), and their dimensions are useless for virus identification and description. UA produces positive staining with large dsDNA-containing particles and not with ssRNA-containing phages, filamentous phages, or dsDNA viruses with low DNA content such as marine phage PM2. Consequently, these viruses cannot be detected by positive staining. In addition, UA causes swelling of purely proteinic structures such as phage tails and empty capsids. UA produces excellent contrast and is particularly suitable for resolution of tail striations. Negatively stained heads are more angular and presumably better preserved than in PT. UA spreads more evenly than PT, but tends to crystallize on grids.

Table 1. Comparison of PT and UA

Parameter	PT	UA
pH	Neutral	Acidic
Crystallizes on grids	Generally not	Yes, often in blades
Acts as a fixative	No	Yes
Type of staining	Negative	Negative or positive
Grid life	1 month	10 years or more
Contrast	Poor to good	Poor to excellent
Negatively stained capsids	Often rounded	Angular
Positively stained capsids		Black, shrunken
Protein structures		Swollen

Both PT and UA form glassy films around particles and act as fixatives. PT-stained preparations are stable for a month without loss of detail. UA-stained grids keep easily for 10 years in grid boxes at room temperature. One of the authors (H.-W. Ackermann) has several grids that are 30 years old and still observable. Because PT and UA are complementary, investigators should always use both of them. Fixation, for example with 2.5% glutaraldehyde, may be necessary for marine phages that cannot be processed immediately and have to travel weeks before examination (Cochlan et al. 1993). The stability of glutaraldehyde- or formaldehyde-fixed material seems to be highly variable. Bacteria (Gundersen et al. 1996) and some viruses have a variable loss rate in fixed samples, whereas the formaldehyde-fixed *Emiliania huxleyi* virus showed reasonably high stability for 2–3 years under storage in the dark (Bratbak et al. 1993). As a rule, we recommend harvesting and preparation on grids of fresh material without any fixation.

d. Electron microscopes and imaging:

The electron-optical resolution of EMs has not improved since the 1960s; all technical improvements made since concern ease of operation, automated focusing, digitalization, and gadgetry. The introduction of digital cameras (and hence digital electron microscopes) has not improved image quality, speed or ease of manipulations, or image recording. On the contrary, it has generated a flurry of gray, contrastless pictures of poor resolution which may irreverently be called "gray pulp." Even company prospects show similar gray pictures. Neither prospects nor instruction manuals provide guidance for contrast improvement. It appears that the various EM and camera companies marketed their products in haste and without knowing how to operate them. The relative failure of digital electron microscopes is largely due to lack of information. Fortunately, image contrast can be greatly improved by manipulation of the camera software, particularly of the histogram governing luminescence (gamma), brightness, and intensity values (Tiekotter and Ackermann 2009). These parameters can be adjusted separately for selected areas, and excellent, high-contrast pictures can be so obtained. A major drawback of CCD cameras is the high price of cameras ($50,000 to $300,000) and printers ($5,000–10,000). This compares poorly with $2,000 for a Philips EM300 conventional camera and $10 for photographic trays and puts digital EM out of reach of most laboratories and institutes.

e. Magnification control:

The specifications of manufacturers cannot be trusted, as they depend on lens setting and local electricity supply. Conventional and digital EMs suffer from the same problem and both must be controlled by means of test specimens. Unfortunately, both catalase crystals and T4 tails have their problems. Catalase crystals may be deformed and it can be difficult to find enough parallel lines of subunits for accurate measurements. In addition, the crystals are soluble in water and tend to dissolve into subunits. T4 tails stained with UA (but not PT) may be stretched beyond their normal length. On the other hand, T4 phages are easy to prepare in a microbiological laboratory and much easier to use than catalase crystals. It remains to see how stable and trustworthy digital cameras are over long periods of operation. Unfortunately, to our knowledge, the adjustment of digital EMs and cameras is lengthy and difficult and may take 2–3 h. It is essentially out of reach for investigators, who depend for adjustments on manufacturers and their technicians.

f. Measurements:

Virus particles on conventional photographic images must be measured after enlargement on prints and never on films, plates, or screens. The reason is that measurements of small particles at low magnification are difficult per se and cannot be accurately extrapolated to high magnification. Digitized particles can be measured directly on the monitor screen. Long flexible phage tails may be measured with special flexible rulers. Investigators should be aware of artifacts, should not measure positively stained particles, and should measure at least 10 particles to obtain statistically meaningful data at sufficiently high magnifications (×250,000 or higher) to minimize measurement errors. Isometric viruses or phage heads should be measured between opposite apices. The literature sometimes includes mentions of phage heads of 30 nm in diameter. This puts tailed phages into the vicinity of polioviruses and is clearly an error. The smallest tailed phages known, podoviruses of the ϕ29 type (Fauquet et al. 2005), have head diameters of 40 nm. All others have heads of 50–60 nm in diameter or larger.

g. Virus detection and identification:

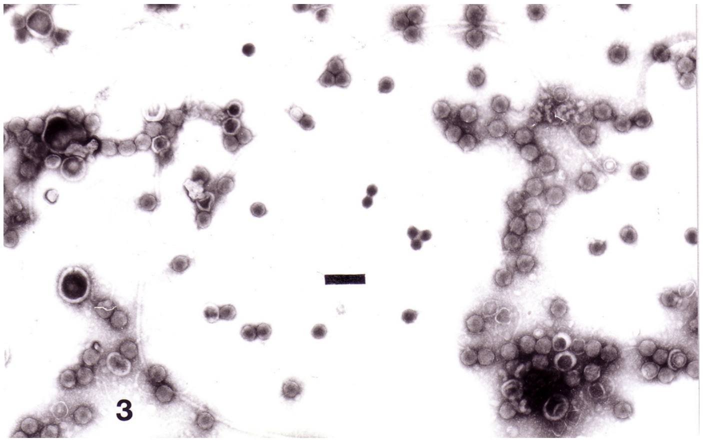
Preparations enriched by incubation with host bacteria may reveal a wide variety of phages or phage-like particles (Rachel et al. 2002). Virus detection is both simple and difficult. It is easy because tailed phages, the mainstay of water samples, are instantly recognizable and differentiated in the EM. They belong to three clear-cut families, *Myoviridae*, *Siphoviridae*, and *Podoviridae* (Ackermann 2007; Ackermann and DuBow 1987; Fauquet et al. 2005). Members of the *Myoviridae* family are characterized by contractile tails like phage T4. Tails are generally thick, rigid, and constituted by a tail tube and a sheath separated from the head by a constriction or "neck." Tail sheaths may be contracted. *Siphoviridae* have long, noncontractile, often very flexible tails like phage lambda. *Podoviridae*, similar to coliphage T7, have short tails of about 10 nm in length. Positively stained podoviruses may appear as headless tails and be difficult or impossible to identify. All tailed phage families have members with isometric or elongated heads. Empty capsids and headless tails will not elicit positive staining and remain invisible.

The detection of isometric, filamentous, or pleomorphic viruses is intrinsically difficult. These viruses seem to be rare in nature, constituting only 4% of prokaryote virus isolates (Ackermann 2007). Further, ssDNA, ssRNA, and small dsRNA viruses do not stain positively. In crude, nonenriched samples, they can be diagnosed (a) after negative staining only and if they (b) occur in large numbers of identical particles, (c) present regular structures such as capsomers, and/or (d) have dimensions corresponding to those of known viruses. The identification of enveloped viruses without inner capsids, such as paramyxoviruses, is difficult or even impossible. Indeed, no pleomorphic virus has ever been identified by simple electron microscopic examination of environmental samples. Even the visualization of spikes on membranous particles is no help, as they also occur on mammalian cell membranes. The detection of filamentous phages is particularly difficult because these viruses are thin, break easily, and may be confounded with pili, flagella, or slime filaments. Algal viruses are isometric or filamentous (Van Etten et al. 1991). Their presence may be suspected if particles are isometric, at least 100 nm in diameter (Fauquet at al. 2005), and show capsomers and transverse edges. Filamentous algal viruses are known in the brown alga, *Chara corallina* (Skotnicki et al. 1976), but should be diagnosed only if they show transverse striations. Pitfalls are many; for example, one of the authors (H.W. Ackermann) once misidentified the mastigoneme filaments of *Ochromonas* algae (Bouck 1971) as possible filamentous viruses. In conclusion, isometric, filamentous, or pleomorphic viruses should be diagnosed only after their concentration in a density gradient or their propagation as pure cultures.

For a definitive diagnosis and identification with known viruses, high-magnification (×250,000 to ×300,000) images and exact dimensions of negatively stained viruses are required. Images of positively stained viruses are unsuitable for this purpose and their dimensions are always inexact (*see* above, 3c; Figs. 4, 5).

h. Artifacts:

Damaged particles and bacterial debris are frequent in phage lysates and environmental samples. For example, tailed phages are quite frequently decapitated, simulating tailless viruses or short-tailed phages. The frequency of tail loss depends on the phage and, possibly, storage conditions. Some



Figs. 3–8. Various bacteriophages and artifacts. Philips EM 300 electron microscope; uranyl acetate (2%, UA) or phosphotungstate (2%, PT) except Fig. 5b, which is a section. Bars indicate 100 nm.

Fig. 3. Negatively and positively (center) stained *Salmonella* phages within the same field; UA, ×92,400.

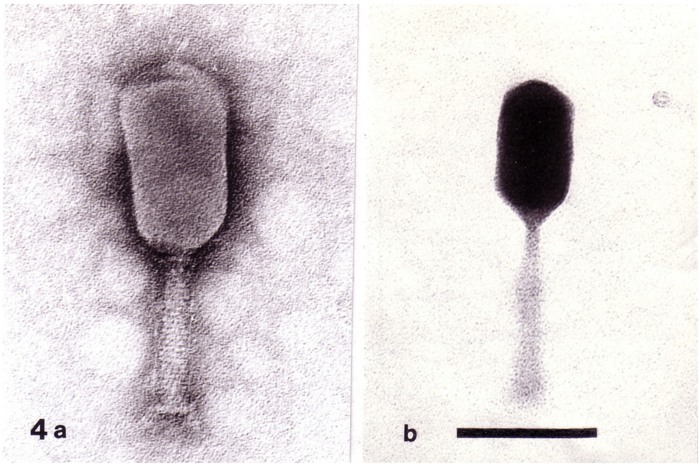


Fig. 4. Negatively and positively (right) stained T4-like *Vibrio parahaemolyticus* phage; UA, ×297,000.

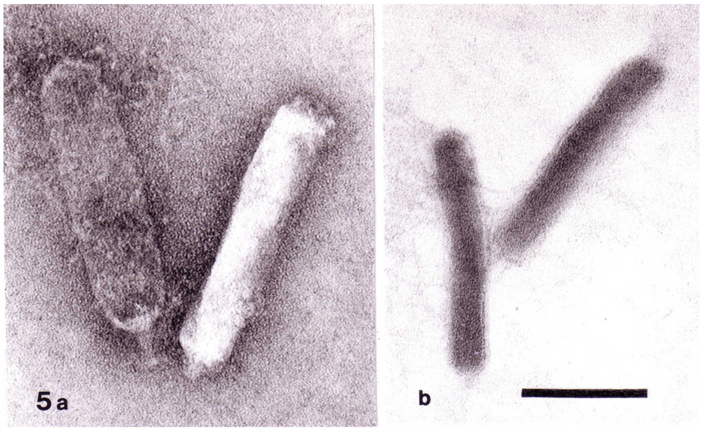


Fig. 5. Negatively and positively (right) stained phage 71A-6 of *Vibrio vulnificus*; UA, ×297,000. The phage is a podovirus with a very long capsid and a barely visible short tail.

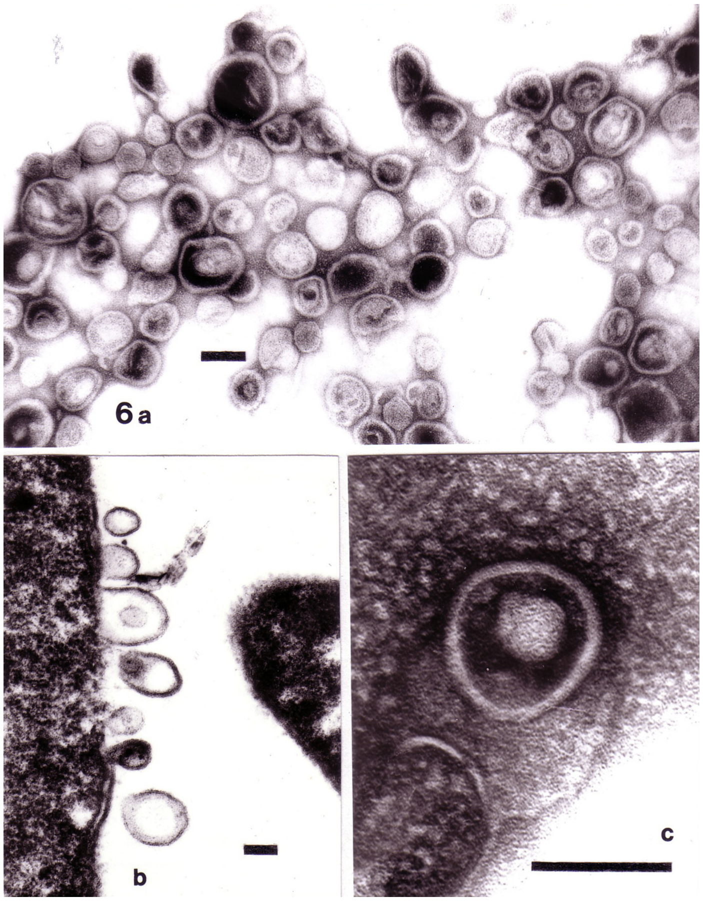


Fig. 6. (a), Cell wall and cytoplasma membrane debris of lysed *Pseudomonas aeruginosa* cells; UA, ×92,400. (b), Vesicles budding from the cell membrane of a phage-infected *Salmonella* bacterium; thin section, ×59,400. (c), False herpesvirus simulated by cell wall debris surrounding an unknown particle; *Vibrio* phage lysate, UA, ×297,000.

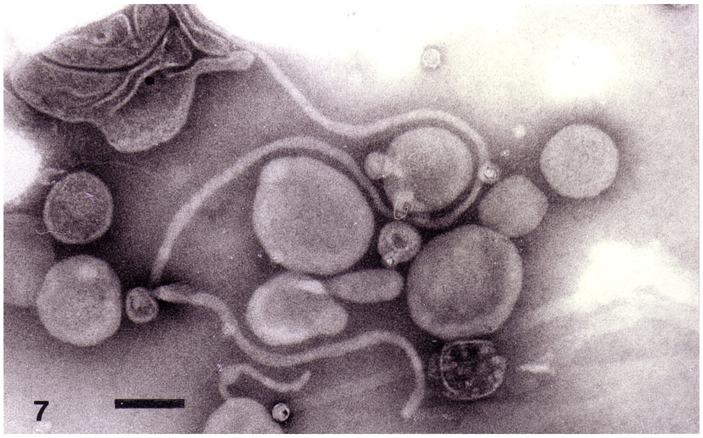


Fig. 7. Slime filament and various bacterial debris. *Bacillus cereus* phage BL1, PT, ×148.500.

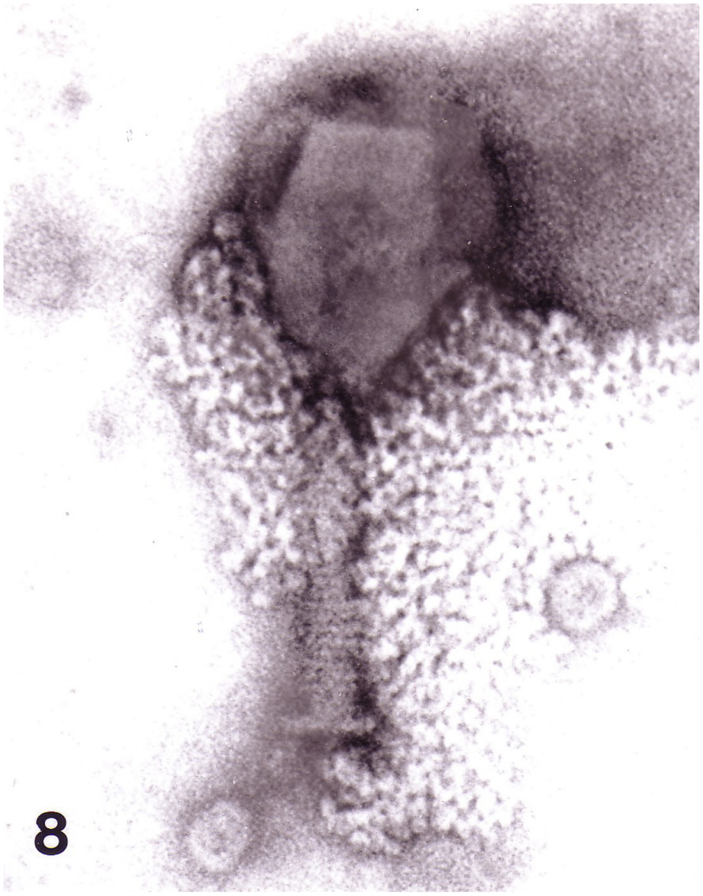


Fig. 8. Phage T4 with a NaCl crystal, UA, ×297,000.

phages are stable over years and some, such as coliphage Mu, fall into pieces within weeks. The frequency of damaged particles increases with repeated freezing and thawing. This should be considered if samples are taken during a maritime expedition, frozen, and processed weeks later. Bacterial debris include round particles derived from cell walls or cytoplasmic membranes, pili, flagella, and capsule-related mucus. Round particles (Fig. 6), often of the size of normal phage heads and thick-shelled, are particularly frequent in lysates of *Pseudomonas* phages, but also occur in those of *Vibrio* and *Synechococcus*. They may be misdiagnosed by inexperienced observers as enveloped viruses. We have even seen round cell debris lodged within other spherical cellular vesicles, simulating enveloped viruses (Fig. 6c) or *Tectiviridae*. All these debris are likely to adsorb DNA. Unless treated with DNase, they are bound to mimic DNA phages in epifluorescence microscopy. Slime, produced for example by xanthan-producing *Xanthomonas* bacteria, is likely to occur in freshwater. When centrifuged, slime blobs may become elongated and then masquerade as filamentous viruses (Fig. 7) and even tailed phages. Short flagella fragments often resemble contractile phage tails, and pili are almost impossible to distinguish from filamentous viruses of the *Inoviridae* family. Further, salts may obscure phage structures (Fig. 8), and UA sometimes simulates envelopes by producing haloes around positively stained viruses (Fig. 9). In conclusion, observers must be wary and constantly on the outlook for artifacts (Table 2).

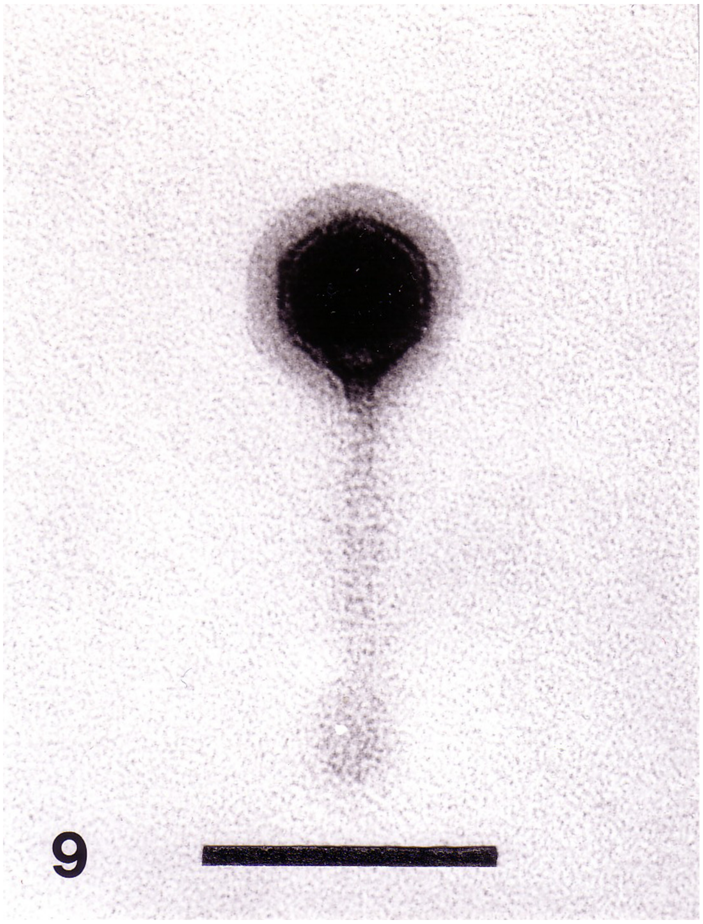


Fig. 9. False envelope in a phage of *Rhizobium* sp.; UA positive staining, ×297,000. Haloes of this type are sometimes seen after UA-positive staining. They expand with prolonged irradiation. Their nature is unclear.

Discussion and conclusions

The investigation of waterborne viruses suffers from the absence of atlases of cyanophages and algal viruses. They should include photographs with scale markers and tables with reliable dimensions for virus identification and comparison. This is a condition for further progress. Uranyl acetate, despite its shortcomings and problems, is irreplaceable simply because it produces good contrast in negative staining and stains viruses positively. At present, it can be replaced only by other uranyl salts. Would more detailed studies of negatively stained preparations improve our knowledge of viral impact on microbial ecosystems? This is certain, but only so if micrographs are of sufficient quality that environmental viruses can be unambiguously linked to (or differentiated from) viruses already known. This is far from being the case.

Table 2. Frequently asked questions.

Can I use grids without a carbon layer?	No. The support film is unstable.
What is better, PT or UA?	Neither. They are different.
What is negative staining for?	Virus descriptions.
What is positive staining for?	Virus counts on grids.
Which viruses can be positively stained?	Those with high dsDNA contents.
What happens to the others?	They are stained negatively or not at all.
Aspect of empty phage heads in PT?	Membranes or black stain-filled vesicles.
Aspect of empty phage heads in UA?	Membranes or shadows.
How to improve virus adhesion to grids?	By glow discharge or wetting agents.
Why should samples be washed?	To remove proteins, sugars, and salts.
Shall I examine unwashed lysates?	Never.
Is fixation necessary?	Generally not.
What is the average diameter of phage heads?	55 to 60 nm.
Do phage heads of 30 nm in diameter exist?	Probably not.
When to suspect large algal viruses (*Phycodnaviridae)*?	In icosahedral, nonenveloped, tailless particles of more than 120 nm in diameter.
How to correct faulty magnification in digital EMs?	By calculation after photographing and measuring phage T4 tails.

Positive staining misses ssDNA viruses, ssRNA viruses, and empty virus capsids entirely and therefore does not give a complete picture of virus populations.

Electron microscopy is time-consuming and still an art. Electron microscopes are as good as their operators, and nothing can replace the eyes and brains of a skilled scientist. Electron microscopy depends critically on the knowledge and dedication of the investigator. It ensues that EM examinations cannot normally be entrusted to a technician or farmed out to another institution. Unfortunately, electron microscopy is taking a bad turn. In former times, using an old-style conventional EM, an electron microscopist could control all parts of a column and adjust astigmatism, resolution, and magnification. The quality and contrast of pictures depended entirely on him or her. This is now less (or not at all) possible with automated and digitized electron microscopes. For any problems of image sharpness and magnification with these instruments, one has to resort to company technicians and to pay them. This can be very expensive, especially if the technicians are located in another city, and success is not guaranteed. The quality of virological, especially bacteriophage, electron microscopy had already sharply declined since 1990 and is much worse than 50 years ago (Ackermann 2004). Presently, with the advent of CCD cameras, dependence on company technicians has become almost total.

This situation is unlikely to reverse itself because (1) digital EMs are popular with inexperienced users, (b) conventional photographical supplies may be difficult to find since the introduction of digital technology, and (c) manufacturers are offering digital EMs only. These instruments may be twice as expensive as conventional EMs and can no longer be adjusted and corrected by users. Their maintenance depends on costly service contracts and company technicians, so underfunded institutions may be tempted to skip maintenance. The final and general consequence is that electron microscopes become concentrated in a few institutions and are accessible for a fee only and sporadically. We fear that environmental scientists will be particularly affected by this trend and we foresee a reduced activity in environmental electron microscopy.

Appendix

Calculation of total virus contents—

The water volume (V) from which particles are harvested during centrifugation may be calculated as follows:

(1) $V = A * (R^2 - r^2)/2R$

where A is the area in cm^2 of the view fields observed on a grid; R and r are the maximum and minimum radius, respectively, in cm of the water in the centrifuge tube and are calculated from the maximum radius of the rotor used (*see* the manufacturer's data sheet), the thickness of the molded flat bottoms, and the height of the water column in the centrifuge tube. The area of the view fields may be calculated as

(2) $A = \pi * (r_{\text{view field}}/\text{mag})^2$

where $\pi = 3.14$, $r_{\text{view field}}$ is the radius of view field of the microscope in cm, i.e., the fluorescent screen, and mag is the magnification. The concentration of particles in the sample (unit is mL^{-1}) is calculated by multiplying the average particle counts per view field with V^{-1}.

Expendable supplies and small equipment (North America only)—

Ernest F. Fullam, 900 Albany Shaker Road, Latham, NY 121110-1491, USA; (800) 833-4024, pdf@fullam.com

Ladd Research Industries, 83 Holly Ct., Williston, VT 05495, USA; (800) 451-3406, ds@laddresearch.com

Ted Pella, 4595 Mountain Lakes Blvd., Redding, CA 96003-1443, USA; (800) 237-3526, sales@tedpella.com

SPI Supplies, P.O. Box 656, 569 East Gay St., West Chester, PA 19381-0656, USA; (800) 242-4774, spi3spi@2spi.com

Soquelec, 5757 Cavendish Blvd. #101, Montreal, QC H4W 2W8, Canada; (514) 482-6427, sales@soquelec.com

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